JAMES LIND, M.D.

(1716 – 1794)

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Every seafarer should recognize the great debt of gratitude owed to James Lind, because out of his numerous studies, observations, and writings came eventually the over-all improvements in diet, hygiene and other public health measures which did so much to improve the vigor and vitality of seamen and to reduce the shocking mortality rates among sailors on long sea voyages.

Although Lind's first great work, "A Treatise on the Scurvy," indicates his outstanding ability in the special field of experimental clinical nutrition, his wide interests, capacity for development and foresight made him above all else a great physician in the very broadest sense. As is the case with many such men, he was years ahead of his time. One hundred years after Lind's death, he was to be praised as one of the "fathers of our modern preventive medicine."

The period of Lind's life covered a large part of the era of great empire expansion. Man's insatiable curiosity, plus his natural lust for adventure, power and possessions led him to undertake long sea voyages to find and claim strange lands and peoples — mostly for the honor of a king or queen far away back home who had little or no interest in the personal safety of the ordinary sailor. This was the day when ships were made of wood and manned by men of steel; when a jail sentence was preferred to service on shipboard, so great was the danger of life at sea.

Scurvy was the principal scourge of seamen —"the great sea plague." Roddis [1] presents a vivid description of the rigors of shipboard life during this period and of the ravages of scurvy among the crews. On Anson's voyage around the

[1] See Acknowledgments, p. 11.

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world (1740–1744), 625 men died out of a total force leaving England of 961 officers and men; most of this loss was due to scurvy. Although this disease did occur at times on shore, particularly during the sieges of war, it was seen most extensively as a shipboard disease. Roddis states that "although no exact statistics are available, it is certain that during that period (1500–1800), scurvy killed as many seamen as were lost by deaths in naval battles, shipwreck, other nautical hazards, and all other diseases affecting the sailor."

James Lind, whose experiments, and the influence of whose writings, would eventually eliminate this dread disease, was born October 4, 1716, in Edinburgh, Scotland. Since Lind's parents were reasonably well off, he had the privilege of a formal education, which in that day provided sound training in the classics. At the age of 15 he was "apprenticed" to a local physician for his early professional training. Not much is known about Lind's early youth or the events of his life during his apprenticeship in medicine. There is little doubt that he carried out the customary duties of the apprentice of preparing drugs and recipes from crude materials then in use, filling prescriptions, and assisting the physician in the duties of his profession. It can be assumed that Lind was exposed to a favorable medical environment, since Edinburgh had already become an excellent and important medical center. Judged by the quality of his later work and publications, he must have been a good student who developed a wide acquaintance with the medical literature of his time.

On completion of his medical apprenticeship in 1739, Lind entered the British navy as a surgeon's mate. While Lind's motives for choosing a career in naval medicine are not completely clear, it might be noted that many young medical men of that time served in the armed forces for reasons similar to those that prevail today. The opportunities for professional experience, travel, the laying away of a "nest egg," and the half-pay allowance given after service of 8 years were undoubtedly factors that influenced his decision. At any rate, it is safe to say that Lind's choice of work was to

lead him ultimately to the top of his profession, to benefit society generally and to produce tremendous advances in the field of naval medicine.

Perhaps a clearer insight into Lind, the man, and his accomplishments may be had if one visualizes the problems and difficulties which confronted a ship's physician in those times. The water-logged oak-hulled ships were musty, evil smelling, rat-infested death traps; always damp, cold and poorly ventilated. Crowding was so great that as many as 900 men had to be housed on board a ship scarcely 180 feet long. The men, sleeping in hammocks strung alternately head to foot, were allowed a space only 14 inches wide per man. Typhus fever, transmitted by the bites of infected body lice, mumps, measles, scarlet fever and other miscellaneous infectious diseases were a common occurrence. During and after naval battles the doctor had an unusually busy time. Splinters from the wooden ships hit by heavy shot were as damaging as shrapnel. Badly shattered limbs were best handled by amputation, as effective methods of limiting and controlling infections were unknown. Despite all of this, scurvy was still the most feared and devastating disease, and Lind was to see much of this.

Now it is of interest to note that up to Lind's time a number of books and papers had been written about scurvy, and in fact many had suggested the use of fruit juices for the prevention and treatment of the disease. It is to Lind's great credit, and an example of his honesty and integrity, that in all of his writing he appreciated this fact and gave due recognition to the earlier observations.

Let us turn back the pages of history to May 25th, 1747, when Lind carried out his experiment which proved conclusively that orange or lemon juice was a specific treatment for scurvy.

Describing his experiment, Lind wrote as follows: "On the 20th of May, 1747, I selected 12 patients in the scurvy, on board the *Salisbury* at sea. Their cases were as similar as I could have them. They all in general had putrid gums, the

spots and lassitude, with weakness of their knees. They lay together in one place, being a proper apartment for the sick in the fore-hold; and had one diet common to all, *viz.* water-gruel sweetened with sugar in the morning; fresh mutton-broth oftentimes for dinner; at other times light puddings, boiled biscuit with sugar, etc., and for supper, barley and raisins, rice and currants, sago and wine, or the like. Two of these were ordered each a quart of cyder a day. Two others took 25 drops of elixer of vitriol 3 times a day, upon an empty stomach; using a gargle strongly acidulated with it for their mouths. Two others took 2 spoonfuls of vinegar 3 times a day, upon an empty stomach; having their gruels and their other food sharpened with vinegar, as also the gargle for their mouth. Two of the worst patients, with the tendons in the ham quite rigid, (a symptom none of the rest had) were put under a course of sea water. Of this they drank half a pint every day, and sometimes more or less, as it operated, by way of gentle physic. Two others had each two oranges and one lemon given them every day. These they ate with greediness, at different times, upon an empty stomach. They continued but six days under this course, having consumed the quantities that could be spared. The 2 remaining patients took the bigness of a nutmeg 3 times a day, of an electary recommended by an hospital-surgeon, made of garlic, mustard-seed, horse-radish, balsam of Peru, and gum myrrh; using for common drink barley-water boiled with tamarinds; by which, with the addition of cream of tartar, they were gently purged 3 or 4 times during the course."

The results of this experiment are depicted just as graphically by Lind: "The consequence was, that the most sudden and visible good effects were perceived from the use of oranges and lemons; one of those who had taken them, being at the end of 6 days fit for duty. The spots were not indeed at that time quite off his body, nor his gums sound; but without any other medicine than a gargle for his mouth he became quite healthy before we came into Plymouth which

was on the 16th of June. The other was the best recovered in his condition; and being now pretty well, was appointed nurse to the rest of the sick.

"Next to oranges, I thought the cyder had the best effects. It was indeed not very sound. However, those who had taken it, were in a fairer way of recovery than the others at the end of the fortnight, which was the length of time all these different courses were continued, except the oranges. The putrifaction of their gums, but especially their lassitude and weakness, were somewhat abated, and their appetite increased by it."

Although Lind might be criticized for the small number of individuals in his experimental groups, his experiment was well planned and the concise and clear statement of his careful observations leaves no doubt as to the significance of his results.

Not only did Lind demonstrate in a controlled experiment the importance of citrus juices in the treatment of scurvy, but his practical and inventive mind led him to develop an improved method for the preservation of citrus juice, a significant development in food technology.

Lind describes his method as follows: "Let the squeezed juice of these fruits be well cleared from the pulp and depurated by standing for some time; then poured off from the gross sediment: or, to have it stay purer, it may be filtered. Let it then be put into any clean open vessel of china or stoneware which should be wider at the top than at the bottom so that there may be the largest surface above to favor the evaporation. For this purpose a china bason or punch bowl is proper; as generally made in the form required. Into this pour the purified juice; and put it into a pan of water come almost to a boil and continue nearly in the state of boiling (with the bason containing the middle of the juice in it) until the juice is found to be the consistency of a thick syrup when cold. The slower the evaporation of the juice the better, and it will require at least 12 to 14 hours continuous in the bath heat, before it is reduced to a proper con-

sistency. It is then, when cold, to be corked up in a bottle for use. Two dozen of good oranges weighing 5 pounds 4 ounces, will yield 1 pound 9 ounces and a half of depurated juice; and when evaporated there will remain about 5 ounces of the rob or extract; which in bulk will be equal to less than 3 ounces of water. So that thus the acid, the virtues of 12 dozens of lemons or oranges, may be put into a quart bottle, and preserved for several years."

It is of interest to note that Lind avoided the use of metal containers to evaporate the citrus juice; even so, some loss of vitamin C must have occurred as the result of this crude process.

Lind had an appreciation for the antiscorbutic values of other fresh fruits and vegetables and sauerkraut, as he recommended their use in his report. He was also acutely aware, as were many physicians, that the severe hardships and exposures to cold and wet on long voyages were important factors in the development of scurvy. In this connection he made strong recommendations for improving living conditions on shipboard. It is of interest to the author that the difficulty of producing experimental scurvy in modern times may be related to the absence of these severe "stress factors" that were so prevalent in Lind's time.

An examination of the standard ration used in the Royal Navy during Lind's day explains the high incidence of scurvy: biscuit, 1 lb. daily; salt beef, 2 lb. twice weekly; dried fish, 2 oz. thrice weekly; butter, 2 oz. thrice weekly; cheese, 4 oz. weekly; peas, 8 oz. 4 days; beer, 1 gal. daily. Not only was this diet devoid of vitamin C, but lacked other vitamins as well. The rations also suffered from long storage under unfavorable conditions.

It is of interest to note the amount of lemon juice rationed to the crew of His Majesty's Ship *Suffolk* on a long voyage to India. On a daily allowance of two-thirds of an ounce of lemon juice mixed with "grog" and sugar, no cases of scurvy were seen during a voyage lasting 23 weeks and one day. Certainly this amount of lemon juice could have provided at

most 10 mg of ascorbic acid per day. This figure is considerably below the recommended daily allowance in use today.

It will perhaps come as a surprise to learn that despite the excellence of Lind's results, his official position in the Royal Navy, and his detailed report in the "Treatise on the Scurvy" published in 1753, it was not until almost 50 years later (1795) that the Admiralty finally gave orders for the compulsory issue of lemon juice to all ships. This "perversity of officialism" and gross governmental inefficiency undoubtedly resulted in a loss of life from scurvy during this period greater than that from all other causes of seamen's deaths put together.

Lind terminated his active service in the navy in 1748 after nearly 10 years of duty. His return to private life permitted him to go once more to the University of Edinburgh where, after submitting his doctoral thesis, he received his degree of Doctor of Medicine. In 1750 Lind became a Fellow of the Royal College of Physicians of Edinburgh and later its treasurer.

From all accounts, Lind undoubtedly led a busy yet pleasant life in his medical practice, yet in the 10-year period 1748–1758 he found time to write and publish his well-known books, the aforementioned "Treatise on the Scurvy" (1753) and "An Essay on Preserving the Health of Seamen in the Royal Navy" (1757). Each of these books enjoyed such popularity as to require three editions. The later book was undoubtedly a most influential factor in Lind's appointment in 1758 as Physician to the Royal Naval Hospital at Haslar, England.

This position demanded great ability and responsibility and carried with it considerable prestige and better than usual financial rewards. From all accounts, Lind was most competent in this post and used it to put into practice many of the advances in hygiene and care of patients that he had so long advocated.

Although Lind must have been extremely occupied with his work at Haslar, he was not done with experimenting.

The problem of obtaining fresh water from sea water plagued men then, as now. Experiments that had been done on the distillation of sea water to obtain fresh water all called for the addition of numerous and sundry ingredients to the distillation mixture, such as herbs, soap, powdered chalk, and so forth.

After a study of this problem, and the development of an ingenious and practical still made from a large kettle used as a distillation pot, a tea kettle for a still head, and a cast and a musket barrel for a condenser, Lind had this to say: "In the year 1761, I was so fortunate as to discover, that the steam arising from boiling sea water was perfectly fresh, and that sea water, simply distilled, without the addition of any ingredient, afforded a water as pure and wholesome as that obtained from the best springs." The term, "I was so fortunate," indicates the tone of modesty which prevails in Lind's writing. Not only did Lind develop this procedure, but he described and recommended the use of a still head and condenser which fitted as a cover over the large copper vessels used in cooking on shipboard. This afforded a most efficient use of the available fuel, a fact which must have been particularly satisfying to this thrifty Scot.

Although Lind had definitely established priority in this discovery, the Parliament in 1771 awarded a gift of 5,000 pounds to another naval surgeon, Irving, for the development of a method of distilling fresh water from sea water. The most interesting aspect of this, and a good insight into the character and generosity of Lind, is indicated by the fact that he served on the official board which appraised the merits of Irving's work.

The breadth and depth of Lind's medical knowledge are made evident in his interest in tropical medicine, which was becoming of greater importance as the tropics were opened by more and more sea travel. Although Lind did not write the first work on tropical medicine, his "Essay on Diseases Incidental to Europeans in Hot Climates," published in 1768, was certainly a real landmark in this subject, full of acute

clinical observations. The importance of this book may be seen by the fact that it went through 6 English editions and one American, plus translations into German, French, and Dutch.

After 25 years of fruitful service as the head of the hospital at Haslar, Lind resigned on June 30, 1783. He must have been happy in the fact that his son succeeded him to the post.

Little is recorded of Lind after his retirement, although he undoubtedly busied himself with the preparation of new editions of his last book. On July 18, 1794, at Gosport, James Lind died. He was buried in St. Mary's Parish Church, Gosport, Hampshire.

Since the world knows little of its greatest men, it is not surprising that very little is known about Lind other than his works. These, however, stamp him indelibly with the mark of greatness, whose stature can only increase with time. Moreover, if a man can be judged by the regard and esteem held for him by his contemporaries, then Lind must have been a fine person. Perhaps most important of all, Lind retained a curious mind and continued to be a student throughout his life.

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WILLARD A. KREHL, PH.D.
Associate Professor of Nutrition, Yale Nutrition Laboratory, New Haven, Conn.

AN UNRECOGNIZED NUTRITIONAL FACTOR (CORNSTARCH) REQUIRED BY THE DUCK[1]

O. NEAL MILLER[2]

Department of Biochemistry and Nutrition, University of Texas, Medical Branch, Galveston

TWO FIGURES

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INTRODUCTION

Many investigators have considered the special nutritional value of the various carbohydrates normally available for preparation of simplified diets in experimental work. Griffith ('41) stated that cornstarch prevented the hemorrhagic kidney syndrome in rats, and later ('42) reported that certain brands of cornstarch protected the young rat from this syndrome of choline deficiency. More recently, Baxter ('47) has extensively investigated the protective action of cornstarch in preventing the hemorrhagic syndrome due to choline deficiency, and was unable definitely to exclude the possibility of other protective factors in starch. There seems to be little doubt that certain carbohydrates favor synthesis of certain B vitamins by the intestinal flora. Couch, Cravens, Elvehjem and Halpin ('48) have shown that dextrins favor synthesis of biotin by intestinal flora, while sucrose and lactose fail to stimulate the intestinal flora of mature fowl. In contrast, Bacigalupo, Couch and Pearson ('50) found that lactose stimulated the intestinal flora to synthesize biotin, riboflavin, and folic acid in the intestine of the mature cotton rat, whereas

[1] Supported in part by a grant from the National Vitamin Foundation, Inc.

[2] Present address: Departments of Biochemistry and Medicine, Tulane University Medical School, New Orleans, Louisiana.

sucrose, fructose or galactose had little stimulatory action. Mannering et al. ('44) have demonstrated that the growth rate of rats receiving suboptimum riboflavin intake was improved by the inclusion of dextrin or cornstarch in the diet. Similar diets characterized by their content of cellulose, sucrose or lactose failed to show the same effect as those containing dextrin or cornstarch. Hall and Sydenstricker ('47) demonstrated the superiority of cornstarch over sucrose in restoring growth of rats fed a 9% casein diet. It has been noted that adding dextrin to a purified diet was more effective than adding sucrose in restoring growth of rats fed a diet deficient in certain amino acids (Krehl et al., '46). According to Mitchell et al. ('37), diets containing 60 to 70% sucrose, fructose or lactose are inferior to those containing glucose in supporting growth of rats receiving a complete diet.

This laboratory became interested in the possibility that cornstarch contains some unidentified nutritional factor as the result of our experience indicating that sucrose would not replace crude dextrose in the purified diets described by Hegsted and Perry ('47). More interesting, however, was the observation that cornstarch supported better growth and development than either sucrose or dextrose in purified diets. Although only a small depression in growth rate was observed by using a sucrose-containing diet, ducklings did not feather normally and were hyperexcitable. It has been previously reported that ducklings receiving a "sucrose diet" have a very high incidence of pancreatic fibrosis, a syndrome which was seldom seen in animals receiving a diet containing cornstarch (Miller and Rigdon, '52a). Evidence will be presented that strongly suggests the existence of a nutritional factor in cornstarch (CSF) which prevents pancreatic fibrosis in the duckling. A preliminary report of these results has been made (Miller and Rigdon, '52b).

EXPERIMENTAL

Newly hatched white Pekin ducklings were placed in heated raised bottom cages and fed diets which were similar to the

purified diets described by Hegsted and Perry ('47). One hundred grams of the basal diet contained the following: (in grams) vitamin-free casein 18, gelatin 10, cellu flour 4, cornstarch [3] 47.45, corn oil 10, salt mixture [4] 4, calcium diphosphate 1, choline 0.3; (in I.U.) vitamin A 1,200, vitamin D 350; (in milligrams) alpha-tocopherol 50; (in micrograms) menadione 100, thiamine hydrochloride 400, pyridoxine hydrochloride 400, riboflavin 800, calcium pantothenate 2,500, nicotinic acid 4,000, biotin 20, and folic acid 100. Daily food consumption and growth records were kept on all birds. At the conclusion of the experiments (4 or 5 weeks), the birds were autopsied and the pancreas examined grossly with respect to color and graded as to the extent of fibrosis on an arbitrary scale of "hardness." Blood sugar measurements were carried out by the method of Somogyi ('45). Blood fructose levels were determined by the method of Roe ('34). Invertase activity of the duodenum was determined on a homogenate of tissue incubated with sucrose in a phosphate buffer at pH 7.0 (the optimum pH) at 37° for 30 minutes. The incubation mixture was deproteinized with a zinc-barium mixture and an aliquot of the supernatant fluid analyzed for reducing sugars. Protease activity was determined on a homogenate of the duodenum by a method described by Todd and Sanford ('41).

RESULTS

Growth rates of ducklings fed the basal diet (containing cornstarch) are compared with those of birds fed a diet containing sucrose or dextrose as the carbohydrate (fig. 1). The dextrose diet and cornstarch diet supported about the same growth rate, but the sucrose diet supported a diminished growth rate by the second week. No striking differences were observed in the average daily food consumption in any of the groups; however, ducklings ate somewhat less of the sucrose diet. The general appearance of ducklings fed the sucrose

[3] Argo.

[4] Hegsted et al. ('41), modified to include zinc chloride 0.025%, cobalt sulfate 0.01% and sodium fluoride 0.005%.

diet was definitely inferior and was characterized by a greasy, unkempt appearance with poor feathering (fig. 2). It seemed possible that this appearance might result from the accumulation of the somewhat sticky sucrose diet on the down. A group of ducklings receiving the basal diet as well as a group

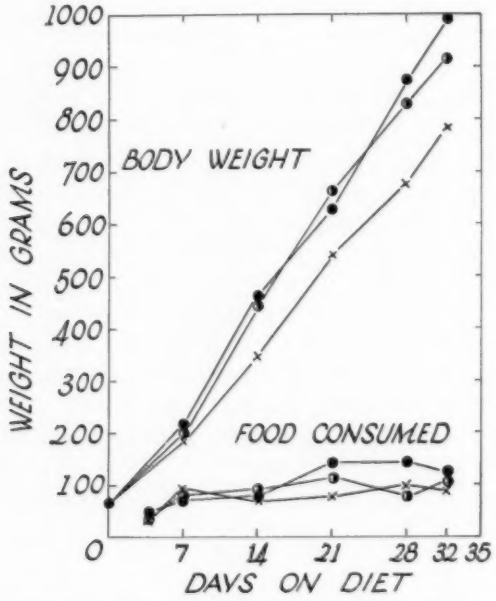

Fig. 1 A comparison of the relationship between carbohydrate in the diet and growth rate as well as food consumption is shown. The mean values of data from 5 to 10 birds are plotted; (●) refers to a cornstarch-containing diet, (◑) to a dextrose-containing diet, and (X) to a sucrose-containing diet.

receiving the sucrose diet were washed on alternate days without marked improvement in the appearance of either group.

The invertase activity of the duodenum was determined, to test the possibility that sucrose was not digested in the intestinal tract and thereby account for the lowered growth rate and inferior appearance due to a chronic caloric de-

ficiency. It was observed that the invertase titre was not impaired in 7 ducklings receiving the sucrose diet, 327 ± 29 as compared to 325 ± 15 mg per gram per hour for 5 ducklings receiving the basal diet. It can be calculated, on the basis of the *in vitro* assay, that ducklings are able to hydrolyze 75 to 100 gm of sucrose over a period of 24 hours, which is ample activity. It is necessary to split only 50 to 60 gm of

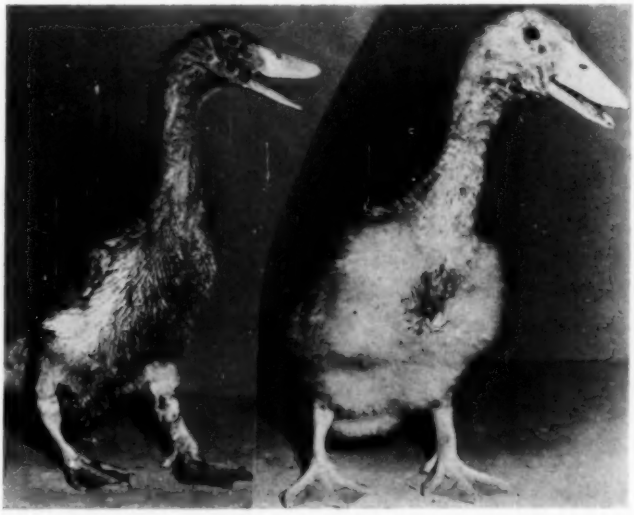

Fig. 2 The duck on the left received a sucrose-containing diet, and the one on the right a cornstarch-containing diet. (See text for description.)

sucrose per day in order to hydrolyze completely the daily intake of sucrose. Undoubtedly the rate *in vivo* is even faster, and these observations definitely demonstrate that sucrose is digested in the duodenum of the duckling.

The high incidence of pancreatic fibrosis suggested a hypofunction of the pancreas. The proteolytic activity of a homogenate of the duodenum (containing pancreatic secretion) was determined. The results of the *in vitro* studies indicated that ducklings (9) which received the basal diet were better able

to hydrolyze the test protein, 6.3 ± 1.0 compared to 4.1 ± 0.4 mg of protein hydrolyzed per gram of tissue per hour. This finding is in contrast to the normal invertase activity and is explained by the well-known fact that invertase is secreted by glands in the intestinal mucosa, whereas enzymes which have digestive activity with proteins in the small intestine are largely secreted by the pancreas.

Hyperexcitability of ducklings fed the experimental diet and the extensive pancreatic lesions suggested a possible hypoinsulinism as well as hypoacinar function. Blood glucose levels were determined on 21 ducklings receiving the basal diet and 14 ducklings receiving the sucrose diet and the levels were 148 ± 5 and 138 ± 9 mg per 100 ml, respectively. These values are not significantly different.

Despite the normal invertase titre in all groups of birds, the possibility remained that the pancreatic lesion may have been the result of unhydrolyzed sucrose being absorbed into the blood stream. Blood fructose levels were determined on 9 ducklings receiving the basal diet and on 10 others which received the sucrose diet. There was more fructose in the blood of ducklings receiving the sucrose diet, 3.8 ± 0.6 as compared to 2.1 ± 0.2 mg per 100 ml, but it is doubtful if the increased fructose level was great enough to cause pathology in the pancreas, particularly in view of the fact that kidneys were normal in all ducklings examined. Two ducklings receiving the basal diet were given intraperitoneal injections of a solution containing 5 gm of sucrose twice daily for 25 days with no untoward effect on the pancreas. In a similar experiment fructose was substituted for sucrose with the same results.

The most logical explanation of the superior growth rate, better feathering, and freedom from pancreatic lesions which characterize the birds fed the basal ration, suggested that better nutrition was afforded the duckling by this diet, in which cornstarch was the carbohydrate. Experiments summarized in table 1 support this viewpoint. It is significant

that commercial feeds [5] appeared to support normal growth and development. The pancreas of ducklings fed this diet were soft, pliable and flesh pink in color. About one-fifth of the ducklings receiving the basal diet had slight hardening of the pancreas, and one-sixth of those receiving the dextrose diet were also observed to have slight hardening of the pancreas. However, when sucrose served as the only carbohydrate in the diet, the hard-pancreas syndrome was observed in 87.0% of the birds. A sucrose diet which included vitamin B_{12} and inositol offered no protection from the pancreatic lesions. Cornstarch was added to the diet to replace 1, 5 or 10% sucrose in the sucrose diet. The inclusion of 1% cornstarch in the diet showed doubtful nutritional benefit; however, increasing the cornstarch to 5 or 10% of the diet protected ducklings from pancreatic involvement.

TABLE 1

The relation of diet to incidence of pancreatic fibrosis

MODIFICATION OF BASAL DIET	NUMBER OF BIRDS	PER CENT INCIDENCE
Control (Purina Startena and Growena)	12	0.0
Basal (containing cornstarch)	31	21.2
Dextrose as the carbohydrate	12	16.6
Sucrose as the carbohydrate	31	87.0
Sucrose with added B_{12} and inositol	5	100.0
Sucrose with added cornstarch (1%)	8	75.0
Sucrose with added cornstarch (5%)	9	55.5
Sucrose with added cornstarch (10%)	5	0.0
Sucrose with added refiners-mud (2%)	10	50.0
Sucrose with added lipid fraction of refiners-mud (1%)	8	87.5
Sucrose with added residue after lipid extraction of refiners-mud (0.5%)	10	50.0
Sucrose with added mother-liquor from 1st crystallization of dextrose (2%)	9	55.5
Sucrose with added mother-liquor (4%)	8	37.5
Sucrose with added DL-methionine (1%)	7	85.5

[5] Such as Purina Startena and Growena.

Dextrose as well as cornstarch seemed to afford better nutrition for the duckling than sucrose, and therefore some by-products of dextrose production from cornstarch were incorporated into the sucrose diets. Both the first mother-liquor from the crystallization of dextrose and refiners-mud were found to be effective in reducing the incidence of pancreatic fibrosis.[6]

In view of the report by Goldberg and Chaikoff ('51), who demonstrated a similar pancreatic lesion in rats given ethionine, 1% DL-methionine was incorporated in the sucrose diet and was found to be ineffective in the prevention of pancreatic fibrosis. The experimental diet apparently contains adequate quantities of methionine sulfur.

DISCUSSION

The data presented clearly demonstrate that purified diets containing only cornstarch as the carbohydrate support better nutrition in the duckling than similar diets containing sucrose. The most plausible explanation of the data presented postulates the existence of a new and yet uncharacterized nutritional factor contained in cornstarch, CSF. However, the possibility is not completely precluded that the data may have resulted from obscure and indirect causes, although the author does not favor this explanation. The better nutrition afforded the duckling fed a dextrose diet compared to that afforded by the sucrose diet suggests that enough CSF may be adsorbed on crude dextrose to protect ducklings from the pancreatic lesion. The cornstarch factor appears to be concentrated in refiners-mud, which, according to Taylor and Nelson ('20), contains about 50% lipid, 11% protein, 30% moisture, and 3.5% ash, as well as 5.5% reducing sugar and other constituents. The mother-liquor from the first crystallization of dextrose also contains protective amounts of CSF.

Evidence from other laboratories also suggests some special nutritional property of cornstarch as compared to sucrose

[6] These materials were kindly furnished by the Corn Products Company of Chicago.

when used as the carbohydrate of simplified purified diets. Baxter ('47) has studied the protective action of cornstarch in the prevention of the hemorrhagic kidney syndrome of choline deficiency. The choline content of cornstarch was too low to account for protective action. Succinylsulfathiazole (2%) was incorporated in a choline-deficient cornstarch diet and did not increase the incidence of hemorrhagic kidney syndrome as would be expected if the flora of the caecum were producing sufficient amounts of choline or other factors to afford protection.

The recent work of Schwarz ('51) suggests that some strains of yeast contain an unrecognized nutritional factor for rats. This material, factor 3, prevents liver necrosis in young rats. Baxter ('47) noted focal liver necrosis in rats also showing the hemorrhagic kidney syndrome, although Schwarz makes no mention of observing the hemorrhagic kidney syndrome in the animals used in his experiments.

It is conceivable that CSF may be identical with either "factor 3" or the protective factor in cornstarch which prevents hemorrhagic kidney in choline-deficient rats.

SUMMARY

Data have been presented which indicate the existence of a new and as yet unidentified factor important for the nutrition of the duckling. This factor (CSF) is found in cornstarch and certain refinery products of dextrose production as well as in crude dextrose itself. A deficiency of this factor manifests itself in the duck by a greasy, unkempt appearance with poor feathering, poor growth rate, and acute pancreatic fibrosis.

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DETERMINATION OF ENDOGENOUS FECAL CALCIUM IN CATTLE BY A SIMPLE ISOTOPE DILUTION METHOD

W. J. VISEK,[1] R. A. MONROE, E. W. SWANSON AND C. L. COMAR
University of Tennessee-Atomic Energy Commission Agricultural Research Program, Oak Ridge

ONE FIGURE

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A method for estimation of endogenous fecal calcium under varying conditions would be most helpful for calculation of the true digestibility of this element, and for determination of net as well as dietary calcium requirements for functions such as maintenance, lactation or growth. Heretofore, endogenous fecal calcium has been determined under fasting conditions, or on diets free of or low in calcium (Mitchell et al., '37; Longwell, '41; Aylward and Blackwood, '36). These techniques impose abnormal conditions and require that the subject be in a postabsorptive state or at the endogenous level of calcium excretion. Such requirements are often difficult to meet, especially in ruminants. In addition, data obtained under conditions of endogenous levels of excretion and transferred to conditions of normal feeding have limited significance even within the same animal, as pointed out by Benedict and Ritzman ('35). Another approach is that of Mitchell and Curzon ('39), Steggerda and Mitchell ('51), and Hegsted et al. ('52), who estimated the total endogenous calcium excretion at zero calcium intake in humans by extrapolation of a regression line relating calcium intake to calcium balance.

[1] Atomic Energy Commission Postdoctoral Fellow.

The difficulties of experimental design and interpretation make this method a tedious undertaking indeed.

This paper describes a direct method for estimation of endogenous fecal calcium similar in principle to that reported by Kleiber et al. ('51) for determination of endogenous fecal phosphorus in dairy cows using P^{32}. Typical results are presented for cattle. The method depends upon the assumption that after daily injection of radiocalcium into the bloodstream the specific activity [2] of endogenous calcium reaching the intestinal contents becomes equal to that of the circulating calcium. This endogenous calcium is diluted with all of the calcium in the gastrointestinal tract of exogenous origin, and the extent of this dilution, as measured by the relative specific activities of the blood and feces, allows direct calculation of the contribution of each calcium source to the fecal calcium. For example, on a calcium-free diet the specific activities of blood and feces would theoretically be equal because only endogenous calcium would be excreted.

METHODS

Non-pregnant, lactating, grade Jersey and Guernsey cows and pure-bred Hereford steers were used in these studies. Cow 1 was a Guernsey in her 310th day of lactation and cows 5, 6, and 7 were Jerseys at 285 days, 109 days, and 63 days of lactation, respectively. Additional information regarding these animals and the rations fed is presented as a part of table 1. All animals were fed adequate diets according to Morrison's ('49) recommendations for at least two months before being placed on experiment. The level of feeding was established at least a week prior to, and remained unchanged during the course of, any experiment, with the exception of calcium and phosphorus intakes, which were altered as a part of the experimental procedure. Details of preliminary conditioning and general procedures were those described by Hansard ('51) and Hansard et al. ('51). The cows were milked

[2] "Specific activity" as used in this paper is defined as the amount of Ca^{45} per unit mass of total calcium in the sample.

twice daily. A venous catheter was used to administer the radioactivity in the form of $Ca^{45}Cl_2$ adjusted to approximately pH 6. In no case was the volume of the daily dose greater than 3 ml, and the total mass of calcium ranged from 3 to 234 mg. The procedures for chemical analysis and radioassay were those described by Comar et al. ('51); feed was analyzed using Association of Official Agricultural Chemists methods ('45).

The procedural details were based upon a preliminary experiment with two dairy cows (1 and 5), data from which are plotted in figure 1 for cow 1. These animals received an intravenous injection of 150 μc of Ca^{45} daily for 15 days. It may be noted that the specific activities of blood and feces reached a plateau and the curves were parallel after the 8th day. This same condition was found on the third day after the last injection and persisted at least until the 10th postinjection day. To determine whether this constancy of ratios of specific activities was altered by the changes in calcium and phosphorus intakes, cow 5 was fed 300 gm of steamed bone meal daily in addition to her regular diet. The results for this animal showed exactly the same trends. In the subsequent studies a 10-day injection period was considered adequate and the endogenous fecal calcium was calculated from samples taken the 4th, 5th, 6th, 7th, and 8th day after the last injection.

The postinjection period was found to be most convenient for the measurements because the injected Ca^{45} disappeared according to the curves of Hansard et al. ('52) and Visek et al. ('53) and resulted in a rapid change of blood Ca^{45} with time during the injection period. Figure 1 illustrates this behavior for the 9th day, where values are given for the blood Ca^{45} immediately after, 8 hours after, 16 hours after, and 24 hours after that day's injection. Thus it can be seen how difficult it would be to get an average blood value during the injection period.

Hansard et al. ('52) have shown that the major fecal excretion of intravenously injected Ca^{45} occurs within 24 hours

TABLE 1

Daily endogenous fecal calcium excretions in cattle as determined by isotope dilution (description of animals, diet, and calcium balance)

ANIMAL	AGE	WT.	DIET [1]	DAILY INTAKE			DAILY Ca OUTPUT			DAILY ENDOGENOUS FECAL CALCIUM	"TRUE DIGESTIBILITY" OF Ca
				Dry matter	P	Ca	Milk	Urine	Feces		
	yr.	*kg*		*kg*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	%
Cow 1	4	309	Normal [2]	6.9 [8]	33	55	5.1	0.33	56 ± 4 [9]	5.0 ± 0.5 [10]	7
Cow 5	8	376	High Ca [3]	8.2	84	150	9.1	0.27	133 ± 2	7.6 ± 0.4	16
Cow 6	8	288	Low Ca [3]	6.6	18	15	6.6	...	15 ± 0.4	5.8 ± 0.2	39
			Normal [4]	7.1	36	28	6.3	0.83	30 ± 2	6.7 ± 0.7	17
			High Ca [5]	7.0	81	120	6.8	0.97	127 ± 7	8.9 ± 1	2
Cow 7	8	350	Low Ca [3]	7.5	20	17	7.4	...	17 ± 0.8	6.0 ± 0.4	35
			Normal [4]	6.5	39	29	9.9	0.46	20 ± 0.8	7.2 ± 0.3	56
			High Ca [5]	7.3	86	120	8.9	0.46	104 ± 5	7.0 ± 0.5	19
Steer 8	1	248	Normal [6]	5.2	19	17		0.40	16 ± 0.6	3.6 ± 0.2	27
			High Ca [7]	4.3	18	47		0.37	41 ± 2	3.6 ± 0.3	20
Steer 9	1	255	Normal [6]	4.9	19	17		0.39	13 ± 0.5	4.5 ± 0.5	50
			High Ca [7]	5.4	19	49		0.35	45 ± 2	5.2 ± 0.5	19

[1] All roughages were sun-cured.

[2] U. S. No. 3 red clover plus concentrate mixture containing 12.9% crude protein. In addition, cow 5 received 300 gm steamed bone meal daily.

[3] U. S. No. 2 orchard grass hay plus equal parts ground oats and corn gluten meal.

[4] U. S. No. 2 orchard grass hay plus concentrate mixture containing 18% crude protein.

[5] Same as above plus 300 gm steamed bone meal daily.

[6] U. S. No. 2 orchard grass hay plus ground yellow corn and soybean oil meal.

[7] Same as above plus 80 gm $CaCO_3$ fed by capsule daily.

[8] All values except otherwise noted represent means of 10 daily observations.

[9] Mean of 10 daily observations ± the standard error.

[10] Mean of 5 daily observations in the postinjection period ± the standard error.

after injection. Therefore, in the calculations, the specific activity of the feces on any one day was compared to the specific activity of the blood on the previous day. Actually, under the experimental conditions used, this time relationship is not critical because the change of specific activities with time is very small.

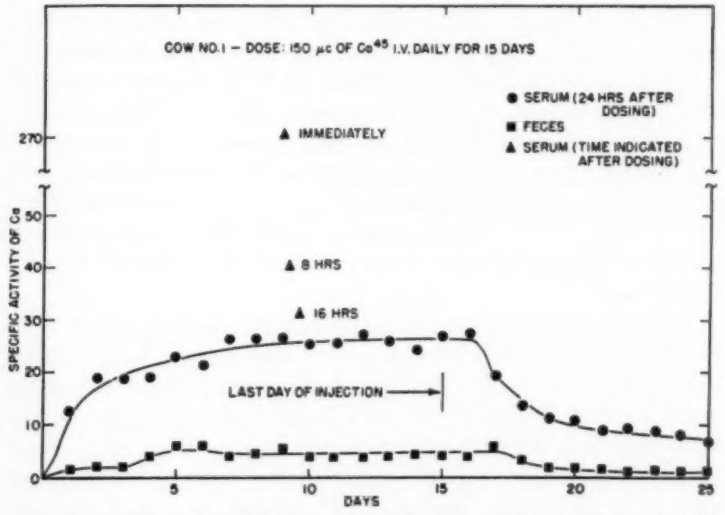

Fig. 1 Specific activities of blood and fecal calcium as observed in a lactating dairy cow during a 15-day injection period followed by a 10-day postinjection period. The values are plotted as a function of time after the first day of injection.

The calculations were made as follows:

$$\frac{\text{Specific activity of fecal calcium on day t}}{\text{Specific activity of blood calcium on day t-1}} \times \text{Daily fecal calcium (gm)} = \text{Endogenous fecal calcium (gm)}.$$

RESULTS

Table 1 presents a description of the animals and diets, the total dry matter, calcium and phosphorus intakes, the calcium losses, the values for daily endogenous fecal calcium excretion and "true digestibility" values. Endogenous fecal

calcium excretions represent the mean of 5 daily observations, whereas all other values are the means of 10 daily observations. The standard deviations are omitted in the interests of economy of space wherever the day-to-day variations did not exceed 5%. Calculations of "true digestibility" were made using average values. In the case of the total fecal calcium excretion, the day-to-day variation was considerably higher than for the dry matter, calcium and phosphorus intakes, and the standard errors are presented to show the correlation between the variation in total calcium excretion and the variation in the endogenous values. Thus, the uncertainty in the estimation of fecal calcium was apparently the limiting factor in the precision of the endogenous values, a fact which is not unexpected from experience in conventional digestibility trials. The standard deviations of the means of 5 daily endogenous fecal values ranged from about 5 to 10% of the mean.

Variations in calcium intake and in the calcium-phosphorus ratio did not appreciably change the endogenous fecal calcium values between the normal and high calcium diets within the same animal. However, in cows 6 and 7 the endogenous fecal excretion was slightly lower on the low calcium diet. More experimental data will be required to show whether or not this is a significant decrease. It should be noted that the calcium intake was varied only over relatively short periods of time. Different results might be expected if the depletion or luxury regime had been in effect for the much longer time period probably necessary to alter the calcium reserve (Hegsted et al., '52).

These studies were not designed to determine the endogenous fecal calcium as a function of age, weight, physiological condition or other factors; nevertheless, certain comparisons can be made. The daily endogenous fecal calcium of the three 8-year-old cows was about 7 gm, whereas the values for the yearling steers were significantly lower ($P = 4.8\%$) at about 4 gm; the value for the 4-year-old cow fell intermediately, at 5 gm. In general, the endogenous fecal value increased with

increasing age, body weight and dry matter intake; however, specific correlations with these variables as well as with the effects of lactation must await further studies.

"True digestibilities" as shown in table 1 were calculated as follows:

$$\frac{\text{Ca intake} - \text{fecal Ca} + \text{end. fecal Ca}}{\text{Ca intake}} \times 100.$$

These values tended to decrease as the dietary calcium intake increased, which would be expected for the ranges of calcium intake used. In view of the dietary variations employed both as to the source and the amount of calcium, the values have limited usefulness under practical conditions. Further studies will be required for comparison of digestibility of calcium as a function of source and as a reflection of the physiological state of the animal.

DISCUSSION

Endogenous calcium may reach the feces by way of the bile, saliva, gastric juice, pancreatic juice, intestinal secretions and other processes (Mitchell and Curzon, '39; Schmidt and Greenberg, '35). The experimental method described in this paper is based on the assumption that the endogenous calcium in the feces, regardless of the pathway, will have the same specific activity as the labeled calcium of the plasma. This assumption is supported by studies which show that labeled calcium introduced into the bloodstream very shortly comes into equilibrium with the calcium in the soft tissues (Plumlee et al., '52; Hansard et al., '52; Thomas et al., '52), and Visek et al. ('53) have shown that radiocalcium added to blood *in vitro* comes to equilibrium with 4 fractions of blood calcium within a matter of minutes. It seems reasonable to assume that equilibrium and steady state conditions obtain certainly by the third postinjection day.

The difficulties in estimating the "true digestibility" or utilization of inorganic materials such as calcium arise primarily because such minerals may be recycled and re-used in the body processes by secretion into the tract at one level and reab-

sorption at another (Bergeim, '26). In this respect they are unlike many organic nutrients which undergo a one-way catabolism and whose utilization may be determined from excreted catabolic end products. In the isotope dilution method, any calcium which reaches the plasma pool before excretion into the feces is regarded as endogenous. The method gives no information as to the pathway by which the endogenous calcium reaches the feces, but only an over-all measure. This method may, of course, be applied to the study of other elements and compounds and, if desired, the results obtained may also be viewed from the standpoint of estimating what fraction of the dietary intake passes through the gastrointestinal tract without being absorbed.

The nutritional importance of calcium is well-recognized. The significance and interrelationship of the numerous factors which contribute to the calcium requirement of animals and an exposition of the difficulties in providing a sound basis for estimation of dietary requirements have been adequately reviewed (Mitchell and McClure, '37; Mitchell and Curzon, '39; Hegsted et al., '52). As pointed out in these reviews, the calcium requirement can be broken down into needs for maintenance, growth, pregnancy and lactation. Growth, pregnancy and lactation requirements can be and have been estimated directly by analysis of product. The maintenance requirement in ruminants, which has not heretofore been estimated directly, is practically equal to the endogenous fecal calcium, since the urinary and miscellaneous losses are relatively small except for the very young, rapidly growing animal. It is now possible to determine directly each component requirement, and a summation thereof should give the net calcium requirement. The isotope dilution method also permits direct estimation of the utilization of dietary calcium, which can be used to calculate the dietary requirement from the net value.

So far as is known, there are no values in the literature which are comparable with those reported in this paper. Aylward and Blackwood ('36) reported an endogenous calcium

excretion of about 2.7 gm daily for two dairy cows fasted from 10 to 12 days; the weights of these animals were not given. This value is somewhat lower than those given here, which is not unexpected as a result of the fasting conditions. It is interesting that the endogenous values for calcium of 4 to 7 gm daily are about half the fecal endogenous phosphorus values of 10 to 14 gm daily reported by Kleiber et al. ('51) for two dairy cows. This relationship is in accordance with the results of other methods (Mitchell and McClure, '37).

The primary value of the method described is that it is simple experimentally and can be applied to give the endogenous fecal value under a wide variety of physiological and nutritional conditions.

SUMMARY

Endogenous fecal calcium was estimated *directly* in *normal* cattle by a method which consisted of 10 daily intravenous injections of radiocalcium, comparison of the specific activities of blood and feces taken on the 4th to 8th day postinjection, and routine determination of calcium balance. The daily endogenous fecal values for three 8-year-old dairy cows averaged 7 gm and for two yearling Hereford steers about 4 gm. The endogenous fecal calcium was not significantly changed when the dietary calcium was varied from normal to a high level. In two animals on a low calcium diet a decrease in endogenous fecal calcium was indicated.

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BACTERIAL CULTURES IN THE NUTRITION OF POULTRY

I. EFFECT OF DIETARY BACTERIAL CULTURES ON THE GROWTH AND CECAL FLORA OF CHICKS [1]

G. W. ANDERSON, S. J. SLINGER AND W. F. PEPPER

Departments of Bacteriology and Poultry Husbandry, Ontario Agricultural College, Guelph, Canada

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Cohendy ('06a) fed pure milk cultures of *B. bulgaricus* to humans and observed a decrease in intestinal putrefaction which was apparent for 7 weeks after treatment. The same worker ('06b) found that after feeding *B. bulgaricus* the organism became thoroughly acclimated in the human intestine and was present for several weeks after ingestion. Belanowsky ('07) fed *B. bulgaricus* to mice and found that this organism became established in the intestinal tract and persisted for some time. In addition, putrefactive types of flora were greatly reduced by such treatment.

Rettger ('15) found that milk and lactose exerted a marked influence on the character of the intestinal bacteria in white rats and chickens. Within a very few days after ingestion of milk or lactose a transformation of the flora took place in which the usual mixed bacterial flora gave way to ones that were more simplified and in which *B. acidophilus* and *B. bifidus* were, as a rule, prominent. This author concluded that the influence of milk was due to its high content of lactose. Essentially the same results were obtained whether sweet or sour milk was fed, and no difference could be observed in

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the the growth- and health-stimulating properties of ordinary sour milk and of milk soured by inoculation with *B. bulgaricus*.

Rettger and Cheplin ('20) found that the daily administration of *B. acidophilus* to white rats caused a marked change in the character of the intestinal flora, with almost a complete transformation to this type of organism. The addition of lactose or dextrin to the basal diet caused a similar change in microflora. The simple character of the new flora reverted to normal gradually with the discontinuance of feeding the organism or the carbohydrates. In contrast to the findings of Cohendy ('06a, b) and Belanowsky ('07), the above workers were unable to influence the intestinal flora of rats by the daily administration of *B. bulgaricus*.

Anderson et al. ('52) reported that the feeding of penicillin to chicks caused an increase in numbers of lactobacillus, aciduric, anaerobic and microaerophilic, aerobic and coliform types of organisms in the ceca. The increase in coliforms was due to the presence of an atypical strain of *E. coli*. In this work dietary penicillin caused a reduction in numbers of the members of the enterococcus group.

The experiments to be reported here were prompted by the finding in our laboratories that certain groups of organisms were increased and others reduced in number by the feeding of penicillin. It was felt that the feeding of such microorganisms might aid in explaining the mechanism of the growth response to antibiotics.

EXPERIMENTAL

Experiment 1

This experiment involved 16 groups of 20 Columbian Rock male chicks. The birds were reared in electrically heated battery brooders with raised wire floors. The experimental diets and tap water were supplied ad libitum for the 5-week duration of the trial. The basal diet contained 60.75% ground yellow corn, 33% soybean oil meal, 2% dehydrated cereal grass,

0.5% ground limestone, 3.25% steamed bone meal, 0.25% iodized salt and 0.25% fish oil (300 I.C.U., D., and 1,500 I.U., A per gram). In addition, 150 mg riboflavin, 900 mg niacin, 300 mg Ca pantothenate, 0.2 mg vitamin B_{12}, 70 gm choline chloride (25%), 150 mg potassium iodide (90%) and 10 gm manganese sulfate (technical) were added to each 100 lb. of diet. This basal diet was fed along with various bacterial cultures in the absence and presence of 10 p.p.m. of procaine penicillin G.

The bacterial cultures used for feeding were isolated from the cecal contents of chicks fed diets containing penicillin. These organisms were maintained as pure cultures on suitable media. Twenty-four-hour-old cultures were mixed with the feed daily at a level of 10 ml of liquid culture to each 100 gm of basal diet. This level of liquid culture supplied 0.08, 0.35 and 1.0% dry matter, respectively, exclusive of microorganisms, when nutrient broth, thioglycollate and skim milk media were used. The number of viable cells fed each day was estimated by the dilution and plate count method. The M.P.N. method was used for tube cultures. An attempt was made to maintain the following numbers of cells per milliliter of liquid culture: atypical *E. coli* strains 1 and 2, 5×10^9; *E. coli*, 5×10^8; micrococci, 5×10^8 and anaerobes, 50×10^{10}.

The birds were weighed individually at weekly intervals and feed consumption was recorded. Fifteen birds (5 per day at two-day intervals) were sacrificed from each group at the end of the 5-week experimental period. Bacteriological analysis and pH determinations were carried out on the cecal contents of these birds. The cecal contents from the 5 birds in each case were pooled, thoroughly mixed and a 1-gm sample plated out in duplicate for bacteriological analysis. The remainder of the pooled cecal contents was used for pH determination. The values presented for bacteriological counts and pH determinations, then, are the average of duplicate determinations made on pooled cecal samples of 5 birds on each of three occasions. The methods employed for pH de-

termination and bacteriological analysis are those described by Anderson et al. ('52).

Results and discussion

The weight and feed efficiency data at 5 weeks of age are presented in table 1. The weights were examined statistically by the "t" test (Snedecor, '46). It will be noted that neither the atypical *E. coli* strain 1 nor penicillin, alone, produced

TABLE 1

Effect of bacterial cultures on chick growth

ADDITIONS TO BASAL DIET	RESULTS AT 5 WEEKS OF AGE					
	Group no.	No penicillin		Group no.	10 p.p.m. penicillin	
		Ave. wt.	Feed/gain		Ave. wt.	Feed/gain
		gm			*gm*	
Nutrient broth media	1	508	2.10	2	520	2.16
N.B. + Atypical *E. coli* 1	3	509	2.41	4	616[2]	2.12
N.B. + Atypical *E. coli* 2	5	555[1]	2.14	6	571[4]	2.12
N.B. + *E. coli*	7	528	2.27	8	586[5]	2.13
Skim milk media	9	547[3]	2.13	10	539	2.13
S.M. + micrococci	11	493	2.12	12	564[6]	2.00
Thioglycollate media	13	547	2.24	14	575	2.01
Thioglycollate + anaerobes	15	549	2.05	16	561	2.07

[1] Significantly greater than 1 ($P < 0.05$).
[2] Significantly greater than 11 ($P < 0.05$).
[3] Significantly greater than 1, 2, 3 ($P < 0.01$).
[4] Significantly greater than 1 ($P < 0.01$) and than 2 ($P < 0.05$).
[5] Significantly greater than 1 ($P < 0.01$) and than 2 and 7 ($P < 0.05$).
[6] Significantly greater than 11 ($P < 0.01$).

a significant growth response. However, this strain of *E. coli* in the presence of penicillin caused a highly significant increase in weight. These results are indicative of a synergistic interaction between the culture of atypical *E. coli* strain 1 and the antibiotic. While strain 1 produced the characteristic green metallic sheen of *E. coli*, it did not ferment sucrose or dulcitol and did not give the standard Imvic's reaction.

The feeding of atypical *E. coli* strain 2 caused a significant increase in weight both in the presence and absence of penicillin. This strain was found to produce an abnormal green metallic sheen, fermented sucrose slowly, produced no gas with rhamnose and gave the standard Imvic's reaction. The feeding of a culture of typical *E. coli* plus penicillin resulted in a weight significantly greater than that of the groups receiving *E. coli* or penicillin, alone. These results indicate a synergistic interaction between this culture and the antibiotic.

Considering groups 9, 10, 11 and 12, it may be noted that the addition of micrococci caused a significant reduction in growth as compared to that obtained with the basal diet. The addition of penicillin to this diet caused no increase in weight, while a combination of the culture of micrococci and the antibiotic resulted in a weight which was significantly greater than that of the group fed the culture alone. The data indicate that penicillin completely relieved the inhibitory effect of dietary micrococci. The micrococci employed were lactic acid producers and the culture was a mixed one made up of two species containing gram-positive cocci, occurring singly and in chains.

A comparison of groups 13, 14, 15 and 16 indicates that neither the feeding of a culture of anaerobes, penicillin or a combination of the two caused a significant increase in growth. However, all treatments caused some improvement in feed efficiency.

The effect of feeding the bacterial cultures and penicillin on the pH and cecal flora is shown in table 2. All bacterial counts were analyzed statistically by the method of analysis of variance (Snedecor, '46). Considering groups 1 to 8, the over-all effect of penicillin was to cause a reduction in pH and in numbers of the organisms studied. Anaerobes, lactobacilli and enterococci were significantly reduced by penicillin, while the reduction in aerobes and coliforms approached significance. An interesting point is that, while the feeding of the various coliforms caused a slight decrease or no

increase in aerobes, anaerobes, coliforms and lactobacilli in the absence of penicillin, such treatment tended to increase the numbers of these organisms in the presence of the antibiotic. These increases were significant in the case of aerobes and anaerobes. It is also apparent that, in the presence of penicillin, enterococci counts were reduced by feeding coliform cultures. In the presence of penicillin, the feeding of coliform cultures tended to increase cecal pH. These increases in pH were accompanied by improved weight of the chicks. This suggests that the lowered pH per se, caused by penicillin, is not responsible for the growth response produced by the antibiotic.

When the culture of micrococci was fed in the absence and presence of penicillin (groups 9 to 12), the antibiotic again caused a reduction in pH and in numbers of the organisms studied. This reduction was significant in the case of aerobes, lactobacilli and enterococci. In general, micrococci exerted no great influence on the organisms considered, but did cause a significant reduction in coliforms in the presence of penicillin. From these results it is difficult to determine why the culture of micrococci caused growth inhibition and how the penicillin completely overcame this deleterious effect.

The results of feeding a culture of anaerobes and penicillin on pH and cecal flora (groups 13 to 16) indicate that, while the over-all effect of penicillin was to cause a decrease in pH and in numbers of the organisms studied, the antibiotic caused a significant increase in coliforms. In the absence of penicillin the feeding of the anaerobic culture tended to increase the numbers of aerobes, anaerobes, coliforms and lactobacilli. Whether these changes are in any way related to the improvement in feed efficiency which resulted from the feeding of anaerobes cannot be stated. It may be significant that, in most cases throughout this work, optimum weights and feed/gain ratios were achieved in groups showing the lowest enterococci counts.

TABLE 2

Effect of bacterial cultures and penicillin on the pH and cecal flora of chicks

GROUP NO.	ADDITIONS TO BASAL DIET	pH	AEROBES	ANAEROBES	COLIFORMS	LACTOBACILLI	ENTEROCOCCI	TREATMENT MEANS
	Effect of feeding coliform cultures							
1	Nutrient broth	6.32	9.04 [1]	10.48	8.73	8.69	8.01	
3	N.B. + Atypical *E. coli* 1	6.09	8.70	10.76	8.00	8.74	8.52	
5	N.B. + Atypical *E. coli* 2	6.43	8.90	10.38	7.49	8.13	7.86	8.78
7	N.B. + *E. coli*	6.15	8.91	10.52	7.53	8.26	7.49	
	General means		8.89	10.54	7.94	8.54	7.97	
2	N.B. + Penicillin	5.56	7.97	9.56	7.26	7.30	7.15	
4	N.B. + Penicillin + Atyp. *E. coli* 1	5.86	8.63	10.37	7.63	7.78	6.66	
6	N.B. + Penicillin + Atyp. *E. coli* 2	5.86	8.69	9.70	7.17	7.41	6.65	8.10
8	N.B. + Penicillin + *E. coli*	5.84	8.67	10.39	8.04	8.01	6.76	
	General means		8.49	10.04	7.53	7.63	6.81	
	Effect of feeding a culture of micrococci							
9	Skim milk	6.25	8.05	10.05	7.21	8.18	8.06	
11	S.M. + Micrococci	6.20	7.48	9.92	7.16	8.12	8.07	8.29
	General means		7.76	9.99	7.19	8.15	8.07	
10	S.M. + Penicillin	5.56	7.25	9.86	7.77	7.21	6.88	
12	S.M. + Penicillin + Micrococci	5.65	7.15	9.86	6.94	7.42	6.88	7.66
	General means		7.20	9.86	7.05	7.32	6.88	
	Effect of feeding a culture of anaerobes							
13	Thioglycollate	6.35	7.44	9.68	6.98	7.68	7.89	
15	Thioglycollate + Anaerobes	6.93	7.87	9.73	7.18	8.08	7.86	8.04
	General means		7.65	9.70	7.08	7.88	7.88	
14	Thioglycollate + Penicillin	5.94	7.51	9.54	7.73	7.38	6.76	
16	Thioglycollate + Penicillin + Anaerobes	6.19	7.58	9.38	7.50	7.33	6.97	7.77
	General means		7.55	9.46	7.61	7.35	6.87	

	Coliforms	Micrococci	Anaerobes
L.S.D. for treatment means	0.18	0.22	0.18
L.S.D. for general means	0.42	0.49	0.41
L.S.D. for average logs of numbers of bacteria	0.85	0.71	0.57

[1] Average log of number of bacteria per gram of cecal contents (wet weight).

Experiment 2

This experiment was made in an attempt to determine whether the activity of coliform cultures was due to the living organisms or to a factor or factors synthesized by the organisms and present in the medium. Ten groups of 20 Columbian Rock chicks of mixed sexes (8 ♀ and 12 ♂) were employed. The chicks were reared in a similar manner and the basal diet was the same as in experiment 1. One group received 10 ml of a liquid culture to each 100 gm of basal diet. The culture in this case was a mixed coliform culture made up of two atypical strains of *E. coli* and the typical *E. coli* used in experiment 1. Other groups received 10 ml of the filtrate or the cells equivalent to 10 ml of the culture per 100 gm of basal diet. The filtrate (supernatant) was obtained by centrifuging nutrient broth containing the mixed culture. No growth was observed after incubation of the filtrate at 37°C. for 24 hours. The cells obtained from centrifuging were washed three times in physiological saline and resuspended in sterile distilled water just before being added to the diets. All diets were fed in the absence and presence of 10 p.p.m. of procaine penicillin G.

Results and discussion

The weight and feed efficiency data are presented in table 3. The weight data were analyzed by the "t" test. Comparing diets 1, 2, 3 and 4, it is evident that penicillin and the mixed coliform culture caused some improvement in weight and feed efficiency. In neither case was the weight increase significant at the 5% probability point. The combination of penicillin and the mixed culture did not, in this case, prove superior to either one alone. Considering groups 1, 2, 5, and 6, it may be noted that the filtrate alone caused no increase in weight, while the filtrate plus penicillin resulted in a weight significantly greater than that obtained with the basal diet (group 1). Comparing groups 7, 8, 9, and 10, it may be seen

that neither the penicillin, the viable cells nor a combination of these resulted in a significant growth response. However, when feed efficiency is also considered it would appear that all treatments gave a favorable response. These data suggest that the activity of the mixed coliform culture may be attributed, in part, to a factor or factors in the filtrate and, in part, to the cells themselves.

TABLE 3

Effect of a mixed coliform culture, the filtrate and cells on chick growth

ADDITIONS TO BASAL DIET	RESULTS AT 5 WEEKS OF AGE					
	Group no.	No penicillin		Group no.	10 p.p.m. penicillin	
		Ave. wt. (♂+♀)	Feed/ gain		Ave. wt. (♂+♀)	Feed/ gain
		gm			*gm*	
Nutrient broth	1	438	2.35	2	484	2.20
N.B. + Mixed culture	3	475	2.30	4	487	2.20
Filtrate	5	424	2.19	6	500 [1]	2.24
None	7	460	2.53	8	483	2.34
Coliform cells	9	474	2.35	10	462	2.33

[1] Significantly greater than 1 and 5 ($P < 0.05$).

Bacteriological analyses and pH determinations were carried out in the manner referred to previously. The results of this work are presented in table 4. The over-all effect of penicillin was to cause a decrease in pH and a reduction in numbers of the organisms studied. This reduction was significant in the case of lactobacilli and enterococci. In the absence of penicillin all organisms except lactobacilli and enterococci tended to be reduced as a result of feeding the coliform culture. In the presence of penicillin, on the other hand, feeding the coliform culture caused increases in all organisms; the increases approached significance in certain cases. These results are similar to the effects noted in experiment 1. In the absence of penicillin, the feeding of coliform cells tended to reduce the numbers of aerobes, anaerobes and enterococci, while in the presence of the antibiotic these organisms were increased as a result of feeding coliform cells. Cecal coliforms

were increased somewhat by feeding the coliform cells both in the absence and presence of penicillin. Feeding of the filtrate tended to cause a decrease in aerobes and anaerobes and an increase in lactobacilli and enterococci, both in the absence and presence of the antibiotic.

The finding in the present experiments that penicillin tended to reduce the numbers of all organisms studied is in contrast to the previous report of Anderson et al. ('52) in which aerobes, anaerobes, coliforms and lactobacilli were increased by dietary penicillin. However, it is noteworthy that in both

TABLE 4

Effect of a mixed coliform culture, the filtrate, cells and penicillin on the pH and cecal flora of chicks

GROUP NO.	DIET	pH	AEROBES	ANAEROBES	COLIFORMS	LACTOBACILLI	ENTEROCOCCI	TREATMENT MEANS
7	Basal	6.04	8.49[1]	10.96	7.39	8.39	7.74	
1	Basal + nutrient broth	6.41	8.73	10.75	7.41	8.57	7.63	
3	Basal + N.B. + coliforms	5.92	8.50	10.29	7.17	8.52	8.32	8.54
5	Basal + filtrate	6.08	8.06	10.53	6.66	8.67	8.55	
9	Basal + coliform cells	6.01	8.30	10.01	7.63	8.59	7.67	
	General means		8.42	10.51	7.25	8.55	7.98	
8	Basal + penicillin	5.75	8.29	10.21	6.99	7.75	7.12	
2	Basal + N.B. + penicillin	5.54	7.81	10.26	6.88	7.53	6.99	
4	Basal + N.B. + coliforms + penicillin	5.68	8.28	10.44	7.46	8.16	8.71	8.17
6	Basal + filtrate + penicillin	5.65	7.68	9.95	7.09	7.72	7.20	
10	Basal + coliform cells + penicillin	5.56	8.50	10.38	7.36	7.49	7.96	
	General means		8.11	10.25	7.15	7.73	7.60	

L.S.D. for treatment means = 0.16.
L.S.D. for general means = 0.36.
L.S.D. for average logs of number of bacteria = 0.80.

[1] Average log of number of bacteria per gram of cecal contents (wet weight).

cases the pH of the cecal contents and the counts of enterococci were consistently reduced by the antibiotic. While the feeding of certain bacterial cultures in the present investigations moderately modified the cecal flora picture, there was no major shift in flora to the organism being fed. These results are interesting in view of the results of Rettger and Cheplin ('20) when *B. acidophilus* was fed to rats.

Romoser et al. ('52) reported that the feeding of viable *A. aerogenes,* isolated from the ceca of chicks fed penicillin, caused slight but consistent growth responses when fed to chicks in the presence or absence of penicillin. These authors concluded that *A. aerogenes* was associated with improved chick growth and appeared to replace in part or enhance the antibiotic response. The present work, which was completed before the appearance of the above report, tends to confirm their findings in principle and indicates that certain other organisms act in a similar manner. It is of interest that organisms of the aerobacter genus have not been encountered in the ceca of chickens in our laboratories.

SUMMARY

Supplementing a diet for chicks with certain microorganisms isolated from the ceca of chicks fed penicillin resulted in improved weight. The feeding of a culture of one atypical strain of *E. coli* caused a significant weight response in the absence of dietary penicillin. The feeding of typical *E. coli* and two atypical strains of *E. coli* in the presence of dietary penicillin resulted in weights considerably greater than those achieved with the microorganisms or penicillin alone. Part of the activity of a mixed coliform culture was found to be present in the filtrate and part in the viable organisms themselves.

A culture of micrococci depressed growth in the absence of penicillin but this inhibition was completely relieved in the presence of dietary penicillin. The feeding of a culture of anaerobes did not improve weight but increased feed efficiency.

There appeared to be a synergistic interaction between penicillin and certain of the bacterial cultures fed.

In most cases dietary penicillin caused a reduction in numbers of aerobes, anaerobes, coliforms, lactobacilli and enterococci in the ceca. The feeding of coliform cultures tended to reduce the counts of these organisms in the absence of penicillin but increased the counts of aerobes, anaerobes, coliforms and lactobacilli, and reduced the counts of enterococci in the presence of penicillin.

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BACTERIAL CULTURES IN THE NUTRITION OF POULTRY

II. EFFECT OF DIETARY COLIFORM CULTURES ON THE GROWTH AND CECAL FLORA OF POULTS [1]

G. W. ANDERSON, S. J. SLINGER, W. F. PEPPER AND M. M. HAUSER
Departments of Bacteriology and Poultry Husbandry, Ontario Agricultural College, Guelph, Canada

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The early work on the feeding of bacterial cultures to various species of animals was reviewed in the preceding paper by Anderson et al. ('53). These workers showed that the feeding of a culture of atypical *E. coli* caused a significant growth response in chicks in the absence of dietary penicillin. Cultures of typical *E. coli* and two atypical strains of *E. coli* fed in the presence of penicillin caused weights considerably greater than those of the chicks receiving the cultures or penicillin alone. Part of the activity of a mixed coliform culture was found to be present in the filtrate and part in the viable organisms. In this work dietary penicillin caused a reduction in numbers of aerobes, anaerobes, coliforms, lactobacilli and enterococci in the ceca. The feeding of coliform cultures tended to reduce the counts of these organisms in the absence of penicillin but increased the numbers of aerobes, anaerobes, coliforms, and lactobacilli, and decreased the counts of enterococci in the presence of penicillin.

The experiments to be reported in this paper were made to determine whether certain of the coliform cultures which

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stimulated the growth of chicks would have a similar effect on poults.

EXPERIMENTAL

Experiment 1

This experiment involved 12 groups of Broad Breasted Bronze poults with 10 ♂ ♂ and 9 ♀ ♀ in each. The poults were reared in electrically heated battery brooders with raised wire floors. The experimental diets and tap water were supplied ad libitum for a 4-week period. The basal diet contained 21.5% ground wheat, 15% ground yellow corn, 5% ground oat groats, 2% dehydrated cereal grass, 2% fish meal, 2% meat meal, 2% dried buttermilk, 45% soybean oil meal, 2.5% ground limestone, 2.0% steamed bone meal, 0.5% iodized salt, and 0.5% fish oil (300 I.C.U., D, and 1,500 I.U., A, per gram). In addition, 150 mg riboflavin, 750 mg niacin, 22.7 gm vitamin B_{12} supplement (12.5 mg B_{12} per pound) and 5.7 gm manganese sulfate (technical) were added to each 100 lb. of diet. The basal diet was fed, along with the bacterial cultures or component parts thereof, in the absence and presence of 10 p.p.m. of procaine penicillin G.

The coliform cultures fed were described in the preceding paper (Anderson et al., '53). Also, the level of culture in the basal diet and the number of organisms in the cultures were the same as indicated in that report. The filtrate and viable organisms of the mixed coliform culture were prepared as indicated in the previous paper. Killed organisms were obtained by boiling the cells for 30 minutes and streaking on nutrient agar to check for complete mortality.

The birds were weighed individually at weekly intervals and feed consumption was recorded. Five birds were sacrificed from the majority of the experimental groups at the end of the 4-week experimental period. Bacteriological analyses and pH determinations were made on the pooled cecal samples from each group, using the methods indicated in the preceding report by Anderson et al. ('53).

Results and discussion

The weight and feed efficiency data at 4 weeks of age are presented in table 1. The weight data for males and females were analyzed separately by the "t" test (Snedecor, '46). All diets containing penicillin gave weights significantly better than the basal diets. Penicillin also improved feed efficiency in all cases. In the absence of penicillin the feeding of the mixed coliform culture caused no improvement in weight. However, comparing groups 4 and 6, it may be noted that in the presence of penicillin the mixed coliform culture caused improved weight in both males and females. These differences, while not significant at the 5% probability point, suggest that there was some interaction between the antibiotic and the mixed culture. Since feed efficiency was not improved, it would appear that, in the presence of penicillin, the mixed culture caused growth stimulation by increasing feed consumption. The filtrate from the mixed coliform culture failed to increase growth either in the absence or presence of the antibiotic.

A comparison of groups 3 and 13 indicates that the culture of typical *E. coli* improved weight in the absence of penicillin. This increase was significant in the case of the females. Feeding the typical *E. coli* culture in the presence of penicillin (compare groups 4 and 14) suggests that the *E. coli* slightly enhanced the activity of the antibiotic. Again, as in the case of the mixed coliform culture, *E. coli* did not improve feed efficiency, so that the culture may have exerted its influence by causing the birds to consume more feed.

Feeding the viable cells from the mixed coliform culture in the absence of dietary penicillin (compare groups 1 and 9) caused a significant reduction in the weight of males. In the presence of the antibiotic, on the other hand (compare groups 2 and 10), the viable cells tended to stimulate the growth of this sex. These data indicate that, in the case of male poults, there was a synergistic interaction between viable mixed coliform organisms and penicillin. The dead organisms from the mixed coliform culture appeared to have

no effect on growth or feed efficiency either in the absence or presence of penicillin.

From the results presented here it is apparent that the mixed coliform culture tended to enhance growth in the presence of penicillin. The activity of this culture appeared to be due mainly to the viable organisms, since neither the filtrate nor the dead cells caused growth stimulation. The lack

TABLE 1

Effect of coliform cultures and the filtrate and organisms from a mixed coliform culture on the growth of poults

GROUP NO.	ADDITIONS TO BASAL DIET	RESULTS AT 4 WEEKS OF AGE			
		Ave. wt. ♂	Ave. wt. ♀	Ave. wt. ♂+♀	Feed/gain
		gm	*gm*	*gm*	
3	Nutrient broth	516	420	468	1.83
4	N.B. + penicillin	717 [1]	597 [1]	657	1.68
5	N.B. + mixed coli	472	436	454	1.98
6	N.B. + mixed coli + penicillin	751 [1]	628 [1]	690	1.72
7	Mixed coli filtrate	494	441	468	1.97
8	Mixed coli filtrate + penicillin	723 [1]	584 [1]	654	1.75
13	N.B. + *E. coli*	559	532 [1]	545	1.85
14	N.B. + *E. coli* + penicillin	697 [1]	642 [1]	670	1.67
1	None	524 [2]	458	491	1.92
2	Penicillin	696 [2]	593 [2]	645	1.71
9	Mixed coli viable cells	445	476	461	1.95
10	Mixed coli viable cells + penicillin	745 [2]	593 [2]	669	1.67
11	Mixed coli killed cells	493	478	485	1.91
12	Mixed coli killed cells + penicillin	666 [2]	605 [2]	636	1.69

[1] Significantly greater than 3 ($P < 0.01$).
[2] Significantly greater than 9 ($P < 0.05$).
[3] Significantly greater than 1 ($P < 0.01$).

of response to the filtrate is somewhat surprising in view of the growth-promoting effect of this fraction in chickens, as reported in the preceding paper. However, the diet used for the poults was designed to be complete in all respects, while the chicken diet was known to be deficient in certain unknown factors present in fish and milk.

The effect of penicillin, the mixed coliform culture, the living and killed cells and the culture of typical *E. coli* on the

pH and cecal floral counts is shown in table 2. Groups 3, 4, 5, 6, 13 and 14 were sacrificed and the determinations made on one occasion and determinations on groups 1, 2, 9, 10, 11 and 12 on a different occasion two days later. Since facilities did not permit more birds to be examined at any one time, and since the filtrate had no effect on growth, groups 7 and 8 were not included in these analyses. The

TABLE 2

Effect of coliform cultures and the viable and killed organisms from a mixed coliform culture on the pH and cecal flora of poults

GROUP NO.	ADDITIONS TO BASAL DIET	LOGS OF NUMBERS OF BACTERIA PER GRAM OF CECAL CONTENTS						
		pH	Aerobes	Anaerobes	Coliforms	Lactobacilli	Aciduric	Enterococci
3	Nutrient broth	5.5	8.22	8.90	7.91	8.32	8.90	6.52
4	N.B. + penicillin	4.8	7.53	8.34	6.74	7.46	7.26	6.52
5	N.B. + mixed coli	5.1	7.18	8.66	6.68	7.69	7.13	5.36
6	N.B. + mixed coli + penicillin	5.0	7.26	8.52	6.22	7.24	6.00	6.36
13	N.B. + *E. coli*	5.6	8.18	9.23	7.06	8.51	8.18	6.69
14	N.B. + *E. coli* + penicillin	5.2	7.34	7.52	6.51	7.38	7.18	5.11
1	None	5.3	6.86	6.66	5.18	7.22	6.71	6.15
2	Penicillin	5.2	6.69	9.36	6.78	6.42	6.60	5.04
9	Mixed coli viable cells	6.4	8.81	8.52	4.90	8.51	8.81	5.69
10	Mixed coli viable cells + penicillin	5.1	5.88	7.69	5.00	6.37	5.30	5.11
11	Mixed coli killed cells	5.6	6.76	8.52	5.60	6.71	6.66	5.69
12	Mixed coli killed cells + penicillin	5.2	7.09	8.52	7.02	7.73	7.08	6.69

bacteriological data were not sufficiently extensive to permit a satisfactory statistical examination.

Again, as in previous work, penicillin consistently reduced the cecal pH. With most diets the effect of penicillin was to cause a reduction in numbers of aerobes, anaerobes, coliforms, lactobacilli, and aciduric types of organisms. While there were some exceptions to this general trend on certain other diets, the main exception was with the groups receiving the killed cells from the mixed coliform culture, where

the antibiotic tended to increase the counts of most organisms studied. Numbers of enterococci were not consistently altered by dietary penicillin.

Feeding the mixed coliform culture in the absence of penicillin (compare groups 3 and 5) caused a reduction in counts of all organisms being considered. This was also true in the presence of the antibiotic (groups 4 and 6) with the exception of the anaerobes, which tended to increase slightly when the mixed culture was fed. The culture of typical *E. coli* did not have a consistent effect on the various groups of organisms in the absence of penicillin. In the presence of the antibiotic, however (compare groups 4 and 14), this culture resulted in decreased counts of all organisms studied, with the greatest reductions occurring in anaerobes and enterococci.

Feeding the viable mixed coliform cells caused an increase in counts of all organisms except coliforms and enterococci in the absence of penicillin (compare groups 1 and 9) and a decrease in all groups excepting enterococci in the presence of the antibiotic (compare 2 and 10). The killed cells caused no consistent effect on the numbers of the various organisms in the absence of penicillin (compare 1 and 11) but increased the counts of all groups except anaerobes in the presence of the antibiotic (compare groups 2 and 12).

From the results obtained in this work it does not appear that the growth-promoting activity of penicillin can be ascribed either to decreases or increases in the groups of organisms investigated. For example, while penicillin decreased the counts with most diets, the majority of organism groups were increased by the antibiotic in the diet containing killed coliform cells. In spite of this, the growth response to penicillin was not materially different in these groups. The most consistent effect of feeding the coliform cultures and the viable coliform cells was the reduction in cecal counts of most of the organisms in the presence of the penicillin. Whether these alterations in intestinal counts were concerned with improved growth cannot be stated. However, it is noteworthy that numerically superior weights were achieved with the mixed coli-

form culture, the culture of typical *E. coli* and the viable cells of mixed coliforms in the presence of penicillin to those obtained with the respective control diets containing only penicillin.

It should be pointed out that there was some incidence of a whitish diarrhea in all groups not receiving penicillin. The diarrhea was completely overcome in every case by including the antibiotic in the diet. The highest incidence of this condition occurred in group 9, where 60% of the males showed signs of the disorder; females appeared less subject to it than males. The depression in growth in the males of group 9 could well have been due to the presence of the diarrhea, and the high pH value and high counts of most cecal organisms encountered in these birds were possibly associated with the disorder. Group 5 males also showed a relatively high incidence of diarrhea and some depression in weight; however, fewer of the affected birds were used in the bacteriological analysis in the case of this group than in group 9.

Experiment 2

In this experiment it was desired to determine the effect of a culture of atypical *E. coli* on the growth of poults. The culture chosen was the atypical strain 2 which was found in the preceding paper of Anderson et al. ('53) to stimulate the growth of chicks. This culture was described in that report. In the present work the culture was grown on skim milk media rather than on nutrient broth. Since it is generally accepted that coliform types of organisms are capable of lactic acid production, other groups of poults were fed 1% of lactic acid to determine whether or not this compound was in any way related to the activity of the coliform culture.

Each group comprised 11 ♂ ♂ and 9 ♀ ♀ Broad Breasted Bronze poults. The birds were fed the same basal diet and reared in the same manner as indicated in experiment 1. Skim milk media or this media inoculated with the atypical *E. coli* was fed at a level of 10 ml of the liquid to 100 gm of feed. All diets were fed in the absence and presence of 10 p.p.m. of procaine penicillin G.

The birds were weighed individually at weekly intervals and feed consumption was recorded. Ten birds were sacrificed from each group (5 per day with a two-day interval) at the end of a 4-week experimental period. Bacteriological analyses were made on the pooled cecal samples from each group on each of the two occasions, using the methods indicated previously.

Results and discussion

The weight and feed efficiency data at 4 weeks of age are presented in table 3. The weight data were examined

TABLE 3

Effect of a coliform culture and lactic acid on the growth of poults

GROUP NO.	ADDITIONS TO BASAL DIET	RESULTS AT 4 WEEKS OF AGE			
		Ave. wt. ♂	Ave. wt. ♀	Ave. wt. ♂+♀	Feed/ gain
		gm	gm	gm	
3	Skim milk media	417	393	405	2.25
4	S.M. + penicillin	581[1]	577[1]	629	1.76
5	S.M. + atypical *E. coli* 2	438	465	452	1.98
6	S.M. + atypical *E. coli* 2 + penicillin	716[1]	557[1]	637	1.76
1	None	420	330	375	2.13
2	Penicillin	700[2]	595[2]	648	1.78
7	1% Lactic acid	429	353	391	2.11
8	1% L.A. + penicillin	684[2]	534[2]	609	1.83

[1] Significantly better than 3 ($P < 0.01$).
[2] Significantly better than 1 ($P < 0.01$).

statistically as in experiment 1. Penicillin caused a highly significant increase in weight and an improvement in feed efficiency in all cases. In the absence of penicillin, the culture of atypical *E. coli* (compare groups 3 and 5) resulted in improved feed efficiency and tended to produce an increase in weight. The weight increase approached significance at the 5% probability level in the females. The coliform culture had little or no effect in the presence of penicillin. The feeding of 1% lactic acid caused a slight but non-significant increase in weight in the absence of penicillin and, if anything, tended to depress growth in the presence of the antibiotic.

The effect of these treatments on the cecal bacteria counts is shown in table 4. The values presented are the average of duplicate determinations made on the pooled cecal contents of 5 birds on each of two occasions. Unlike the results of experiment 1, where penicillin tended to reduce the cecal counts of most of the organisms studied, the effect of penicillin here was extremely variable. Some groups of organisms appeared to be increased and others decreased by the antibiotic but there was little consistency from diet to diet.

TABLE 4

Effect of a coliform culture and lactic acid on the cecal flora of poults

GROUP NO.	ADDITIONS TO BASAL DIET	LOGS OF NUMBERS OF BACTERIA PER GRAM OF CECAL CONTENTS					
		Aerobes	Anaerobes	Coliforms	Lactobacilli	Aciduric	Enterococci
3	Skim milk media	7.37	8.02	6.93	7.29	6.65	5.66
4	S.M. + penicillin	6.96	8.68	6.94	6.62	6.00	5.66
5	S.M. + atypical *E. coli* 2	7.72	8.34	6.66	7.82	7.61	6.36
6	S.M. + atypical *E. coli* 2 + penicillin	7.99	8.55	7.68	7.95	6.60	5.75
1	None	7.14	7.75	6.63	6.99	nil	5.52
2	Penicillin	6.83	8.53	6.45	6.86	5.44	<5.41
7	1% Lactic acid	7.25	8.52	6.27	7.46	6.59	5.56
8	1% L.A. + penicillin	7.51	8.37	7.24	7.62	7.16	5.65

Feeding the culture of atypical *E. coli* in the absence of penicillin (compare groups 3 and 5) tended to increase the counts of all organisms excepting coliforms, while in the presence of penicillin the coliform culture increased the numbers of all organisms with the exception of anaerobes (compare groups 4 and 6). These results are in contrast to the findings in experiment 1, in which case the coliform cultures tended to decrease the numbers of most organisms, particularly in the presence of the antibiotic. A point of interest in this work is that the feeding of 1% of lactic acid influenced the cecal microflora in exactly the same manner as did the culture of atypical *E. coli*. That is, in the absence of penicillin lactic acid caused an increase in the counts of all or-

ganisms excepting coliforms, and in the presence of the antibiotic the acid caused an increase in all groups with the exception of anaerobes. Thus it would appear that the influence of this particular culture on the cecal microflora may be due, in part, to the production of lactic and other acids.

SUMMARY

Broad Breasted Bronze poults were fed a practical diet supplemented with certain coliform cultures isolated from the cecal contents of chicks receiving penicillin. Feeding a mixed coliform culture or the viable cells from this culture resulted in improved weight of the poults when the diet contained penicillin. The weight increases were not significant at the 5% point. Neither the killed organisms nor the filtrate from this culture influenced poult weight. The results suggest that there was interaction between the viable mixed coliform organisms and penicillin.

A culture of typical *E. coli* caused a significant improvement in the weight of female poults in the absence of dietary penicillin and appeared to enhance slightly the activity of penicillin when fed along with the antibiotic.

In a further experiment a culture of atypical *E. coli* resulted in a weight response in female poults which approached significance at the 5% point. This culture did not influence weight when fed along with penicillin. Inclusion of 1% lactic acid in the diet caused a slight, but non-significant, increase in weight in the absence of penicillin and tended to depress growth in the presence of the antibiotic.

No consistent alterations in the cecal floral counts of aerobes, anaerobes, coliforms, lactobacilli, aciduric or enterococci types of organisms appeared to result from feeding penicillin or the various coliform cultures. Nor did the growth-promoting activity of penicillin and coliform cultures appear to be explicable simply on the basis of increases or decreases in these groups of organisms.

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STUDIES ON CAROTENOID METABOLISM

XII. THE EFFECT OF DIETARY CAROTENOIDS ON THE CAROTENOID DISTRIBUTION IN THE TISSUES OF CHICKENS [1,2]

J. GANGULY, J. W. MEHL AND H. J. DEUEL, JR.

Department of Biochemistry and Nutrition, University of Southern California, Los Angeles

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INTRODUCTION

The classical studies of Palmer and associates ('14, '19a, b, c, '22) were devoted to the carotenoid distribution in the tissues of animals on low and high carotenoid-containing diets. On the other hand, Zechmeister and his associates, particularly Tuzson, concerned themselves with the isolation and characterization of pure carotenoids from the tissues of animals obtained from their natural habitat. Their earlier work has been summarized by Zechmeister ('37), while most of the relevant information is assembled in a recent monograph by Deuel ('51). Very little work, however, has been reported dealing with the effect of the direct administration of pure carotenoids on carotenoid-depleted animals.

In the current investigation, the effect of the administration of pure carotenoids on the composition of the various tissues of carotenoid-depleted chickens was followed.

EXPERIMENTAL

General procedures and diets

All the chickens used were New Hampshire Red females. Day-old baby chicks and laying hens were employed.[3] The

[1] This work was assisted by a grant from the Nutrition Foundation.

[2] Contribution 327 of the Department of Biochemistry and Nutrition, University of Southern California.

[3] The day-old chicks were obtained from the Pioneer Hatchery, Petaluma, California, and the laying hens were obtained locally.

laying hens, as indicated by their composition and by that of their eggs, had been receiving a diet high in carotenoids previous to their arrival in the laboratory. This is designated as "farm diet." Its composition is unknown.

Diets 1 and 2, which are low in carotenoids, were fed to the laying hens for periods of from 7 to 160 days, to deplete them as completely as possible of tissue carotenoids. The same diet

TABLE 1

Basal diets used in chick studies

CONSTITUENTS	DIET 1	DIET 2
	%	%
Whole wheat (ground)	40.0	...
Barley (ground)	38.0	...
Commercial casein	11.98	12.0
Starch	..	72.96
Brewers' yeast (Anheuser-Busch, Strain G)	5.0	10
Cottonseed oil (Wesson)[1]	2.0	2.0
Commercial NaCl containing 0.02% KI	1.0	1.0
Salt mixture	2.0[2]	2.0[3]
Choline chloride	0.02	0.02
Manganese sulfate	..	0.02

[1] Containing the following substances per 2 gm oil (100 gm food): vitamin A (Nopco), 700 I.U.; vitamin D_3 (Desynon), 240 chick units; mixed tocopherols, 30 mg.

[2] Composition/100 gm: $CaCO_3$, 30 gm; KH_2PO_4, 30 gm; $CaHPO_4$, 25 gm; $MgSO_4 \cdot 7H_2O$, 9.9 gm; ferric citrate, 4.9 gm; $MnSO_4 \cdot 4H_2O$, 0.1 gm; $ZnCl_2$, 0.05 gm; $CuSO_4 \cdot 5H_2O$, 0.05 gm.

[3] Composition/100 gm: $CaCO_3$, 30 gm; KH_2PO_4, 30 gm; $CaHPO_4$, 25 gm; $MgSO_4 \cdot 7H_2O$, 10 gm; ferric citrate, 4.9 gm; $ZnCl_2$, 0.05 gm; $CuSO_4 \cdot 5H_2O$, 0.05 gm.

was fed to the day-old chicks, and one group was raised solely on this regimen. The young chickens grew fairly well on both diets 1 and 2, and gave eggs when mature, although somewhat irregularly. Likewise, the laying hens receiving the carotenoid-low diet continued to lay eggs. The composition of diets 1 and 2 is indicated in table 1.

Diet 1 is that used by Greenberg et al. ('49) in their vitamin A bioassays, except that vitamin A is included in the present

diet. Diet 1 was shown to contain appreciable amounts of lutein (75 μg/100 gm diet).

Sources of carotenoids

Pure α- and β-carotenes, as well as the 90% β-, 10% α-carotene, were obtained from a commercial source.[4] The pure samples of lycopene and zeaxanthin were kindly supplied by Professor L. Zechmeister of the California Institute of Technology, while the crytoxanthin sample was furnished by Dr. S. M. Patel.

Procedure

The carotenoids were fed in a peanut oil solution mixed with the basal diet. The sample to be tested was dissolved in peroxide-free diethyl ether, and peanut oil was added in the proportion of 1 gm to each milligram of carotenoid. Most of the ether was removed under gentle suction at a low temperature, while the residual quantity was blown out by bubbling nitrogen through the oil solution. This fat solution of carotenoid was mixed with the requisite amounts of the basal diet. Usually 20 gm of the diet mixture contained the daily dose of the carotenoid. The birds were placed in individual cages, were fasted overnight, and were then given the diet mixture containing the carotenoid. No additional diet was given during the period of supplementation. The diet containing the supplement was always eaten up practically quantitatively each day.

The chickens were sacrificed 48 hours after the last supplement had been given. While they were under a light nembutal anesthesia, the birds were bled from the heart; ammonium oxalate was used as an anticoagulant. Carotenoid and vitamin A determinations were carried out on the oxalated plasma. Immediately after removal of the blood, the tissues were excised. These included the liver, abdominal fat and ovaries (in the mature birds). The yolks of eggs present in the hens, or laid eggs, were separated as completely as possible from the whites; they were weighed and used for the analyses.

[4] General Biochemicals, Inc., Chagrin Falls, Ohio.

Extraction of tissues

Blood was extracted according to the method of Kimble ('39). Other tissues were usually saponified with potassium hydroxide and 95% ethanol (1 ml of 50% potassium hydroxide and 2 ml of ethanol per gram of tissue). The non-saponifiable residue was removed by extracting it three times with peroxide-free diethyl ether. The ether extract was washed three times with distilled water and was dried over anhydrous sodium sulfate.

Although the above procedure was satisfactory when the tissues contained small amounts of carotenoids, considerable losses of carotenols occurred during washing when greater amounts of these pigments were present. In this case, the following alternative procedure was employed: The tissues were first digested with 5% potassium hydroxide, and were extracted with alcohol-ether according to the method of Davies ('33). The residue from the ether extract was then saponified. When proper care was used, no loss of carotenols was encountered during this procedure.

In the experiments in which the distribution of free, mono- or di-esters of carotenols was studied, the extraction procedure described by Thompson et al. ('49) was employed.

Chromatography

All tissue extracts were transferred to petroleum ether (b.p., 60 to 70°C.) for chromatography. The chromatographic technic employed was a modification of that used by Thompson et al. ('49), and has been partly described in a recent report by Ganguly, Krinsky, Mehl, and Deuel ('52b). Different batches of alumina varied considerably in activity, and each batch had to be tested before use. In general, for routine work, 2 to 4% acetone was used for the removal of α- or β-carotene, or both, 20 to 35% acetone for vitamin A alcohol or cryptoxanthin, or both, and 8% ethanol for lutein and zeaxanthin. Recovery tests with mixtures of the several carotenoids showed that they can be quantitatively separated by this procedure. When lyco-

pene was present, it was separated with 5 to 10% acetone. For further separation of the α- and β-carotene in the 2 to 4% acetone fraction, a column of magnesia-celite (1:1) was used. For the characterization of α- and β-carotene, when necessary, mixed chromatograms with pure α- or β-carotene were made on similar magnesia-celite columns. No further fractionation was made on the 8% ethanol fraction containing the dihydroxy-carotenoids.

Determination of vitamin A and carotenoids

Vitamin A was determined by the Carr-Price reaction; a Coleman Junior Photoelectric Spectrophotometer calibrated with a standard vitamin A solution was used. The vitamin A alcohol fractions from the tissues of animals receiving the farm diets or supplements of cryptoxanthin contained small but appreciable amounts of carotenoids, for which corrections were applied. However, this fraction from the carotenoid-depleted animals was essentially carotenoid-free, even after the administration of pure carotenoid supplements, so that no corrections were necessary.

Carotenoids were determined at the appropriate absorption peak with a Beckman Photoelectric Spectrophotometer, Model DU. In the first tests, the determinations were made in *n*-hexane. However, in the later tests, the carotenoids were determined in the respective eluents as collected from the chromatographic column, and the concentrations were calculated from the following extinction values ($E^{1\%}_{1\,cm}$) at the coresponding $\lambda_{max.}$: α-carotene, 2,720 (444–446 mμ); β-carotene, 2,600 (448–450 mμ); lycopene, 3,470 (470–472 mμ); cryptoxanthin, 2,530 (448–450 mμ); lutein, 2,520 (444–446 mμ); and zeaxanthin, 2,520 (448–450 mμ). There were slight variations of $\lambda_{max.}$ in the crude fractions of the tissue extracts. During analysis, unnecessary exposure of the carotenoid samples to bright light and to high temperatures was avoided. The carotenoid supplements were given to the animals in a darkened portion of the room.

TABLE 2

The carotenoid and vitamin A content of the blood, liver, ovary, and body fat of hens on farm diet, on diet 1 (carotene-free) or diet 2 (carotenoid-free) for various periods of time [1]

DIET	PERIOD ON DIET	NUMBER OF TESTS	β-CAROTENE				LUTEIN				VITAMIN A			
			Blood	Liver	Ovary	Body fat	Blood	Liver	Ovary	Body fat	Blood	Liver	Ovary	Body fat
	days													
Farm diet	..	4	4.2(3)	1.3	0.8(3)	0.2	162(3)[2]	7.6	35.1(3)	6.8(3)	82(2)	262(3)	6.0(3)	3.3(2)
Diet 1	7	1	0	0.7	0.4	[3]	181	7.2	3.6	13.5	.	..	..	..
	42	1	0	0	0	[3]	32	0.4	0.8	3.6	151	136	0.9	1.8
Diet 2	10	1	1.3	0.2	0.1	3.0	89	3.8	4.2	3.9	84	248	27.0	11.0
	14	1	0	0.6	1.1	0.2	129	4.1	13.3	4.5	131	348	5.0	2.2
	30	1	0	0	0	..	0	1.6	1.0	..	55	227	0.7	..
	160[4]	3	0(1)	0	0	0	0(1)	0.1	0	0.1	91(1)	14.0	2.9(2)	2.6
Diet 1 and then Diet 2	90 / 7	1	0	0	0	[3]	0	0.5	1.3	1.1	104	200	7.2	4.0

[1] Figures in parentheses indicate the number of tests when different from value given in column 3.

Values of vitamin A are in I.U./100 ml plasma or I.U./gm tissue. Carotenoids are expressed as μg/100 ml plasma, or μg/gm tissue.

[2] Solvent n-hexane.

[3] Trace.

[4] Chicks raised on diet 2.

RESULTS

I. Changes in the concentrations of carotenoids in the tissues of chickens on low-carotenoid or carotenoid-free diets

On the farm diet, the hens stored more lutein in the ovaries than in the liver, but the concentration of this carotenoid was reduced more rapidly in the ovaries than in the liver by the carotenoid-free diet. Small amounts of lutein were invariably found in the body fat. The blood became free from lutein earlier than did the liver. This is contrary to the behavior of vitamin A, where blood tends to maintain its normal level even when all vitamin A has been removed from the liver (Ganguly and Krinsky, '53). This discrepancy may be indicative of the physiological importance of vitamin A and of the lack of such function in the case of lutein. These results are summarized in table 2.

II. Variations in the concentrations of carotenoids and vitamin A in egg yolk, as influenced by diet

Diet has been shown to have a profound effect upon the carotenoid content of the egg yolk. Although the eggs obtained from hens on the farm diet contained definite amounts of β-carotene and high concentrations of lutein, no β-carotene was found in the eggs of chickens on diet 2, and the lutein content was decreased to a minimum value. These data are recorded in table 3.

III. Appearance of different carotenoids in the tissues of carotenoid-depleted chickens following the oral administration of pure carotenoids

The concentrations of α-carotene, β-carotene, lycopene, cryptoxanthin, zeaxanthin, lutein and vitamin A in the blood and liver of chickens receiving the carotenoids by mouth are included in table 4, while the concentrations in the ovary are given in table 5.

Only the carotenoids which had been administered appeared in the blood and tissues, as determined by analysis, and so only these values are recorded. Only the hydroxy carotenoids (cryptoxanthin, zeaxanthin and lutein) occurred in the blood and were deposited in the liver and ovaries in appreciable amounts. On the other hand, the carotenoid hydrocarbons (α- and β-carotenes, lycopene) appeared only in traces.

Only unesterified zeaxanthin was found in the blood of all three chickens to which it had been fed and in the ovary of the

TABLE 3

Concentration of carotenoids and vitamin A in eggs as influenced by diet

DIET	DESCRIPTION OF ANIMALS	NUMBER OF TESTS	AVERAGE COMPOSITION OF EGG YOLK [1]		
			β-Carotene	Lutein	Vitamin A
			μg/gm	*μg/gm*	*I.U./gm*
Farm	From hens immediately after purchase from farmer	6	1.2 [2] (0.6–1.8)	26.5 (21.9–33.8)	9.1 (5.5–14.0)
2	From hens previously on carotenoid-free diet for 77 days	4	0	1.0 (0.9–1.1)	4.2 (3.5–5.0)
2	From hens raised to maturity on carotenoid-free diet from 1-day-old chicks	6	0	1.2 (0.4–1.9)	3.8 (2.8–5.0)

[1] Including range of values in parentheses.

[2] Proved to be β-carotene by mixed chromatograms and extinction curves in *n*-hexane and carbon disulfide.

single chicken in which this organ was present. On the other hand, although it was exclusively in the free form in the livers of two birds, 8% of it was present as the di-ester, 14% as the mono-ester, and 78% was free in the liver of the third chicken.

IV. Effect of intravenous injection of colloidal carotenoids upon their presence in the tissues of carotenoid-depleted chickens

Colloidal carotenoids prepared as described by Thompson et al. ('49) were injected intravenously into carotenoid-depleted

TABLE 4

The carotenoid and vitamin A content of the blood and liver of young chickens and hens on carotenoid-low or carotenoid-free diets after the feeding of cartenoids or vitamin A

SUPPLEMENT FED	DIET FED	NUMBER OF TESTS	SUBSTANCE FED		LUTEIN		VITAMIN A	
			Blood plasma	Liver	Blood plasma	Liver	Blood plasma	Liver
			μg/100 ml	*μg/gm*	*μg/100 ml*	*μg/gm*	*I.U./100 ml*	*I.U./gm*
Lutein (0.75 μg/gm food)[1]	1	2	55	0.2	55	0.2	99	22
Vitamin A (7,700 I.U./7 days)	1	2	..	..	46	0.3	184	1,170
.....	2	2	0	0	0	0	110	52
β-Carotene (14 mg/7 days)	1	2	0	0.4	49	0.2	118	348
β-Carotene (7 mg/3 days)	2	6	0	1.2	0	0	136	206
β-Carotene, 90%; α-carotene, 10% (7 mg/3 days)	2	6	0	0.6[4]	0	0	169	178
(7 mg/3 days)[2]	2	2	0	1.2[4]	0	0	113	160
α-Carotene (7 mg/3 days)	2	10	0	1.6	0	0	116	106
α-Carotene (6 mg/3 days)[2]	2	5	Trace	1.2	0	0	87	133
Lycopene (24 mg/12 days)[2]	2	4	7.2	1.4	0	0	..	...
Cryptoxanthin (7 mg/3 days)	2	2	213	5.4	0[5]	0[5]	..	138
Zeaxanthin (14 mg/7 days)	1	2	184[3]	2.6[3]	..	..	108	44
Zeaxanthin (7 mg/3 days)[2]	2	6	526	3.4	..	..	90	..

[1] No supplement given. Diet 1 contains this small amount of lutein.
[2] Experiments on hens. Other tests were on young chicks.
[3] Corrected for lutein content by control values.
[4] β-Carotene; also contains traces of α-carotene.
[5] No dihydroxy-carotenoids present.

chickens, and the birds were sacrificed by heart puncture 24 hours after the last injection. The results are summarized in table 6.

None of the carotenoids was present in the blood 24 hours after they had been administered intravenously in colloidal

TABLE 5

The carotenoids in the ovaries of chickens after the administration of supplements to birds on diet 2

SUPPLEMENT	NUMBER OF TESTS	CONCENTRATION PER GM OF TISSUE		
		Substance fed	Lutein	Vitamin A
		μg/gm	*μg/gm*	*I.U./gm*
No supplement (Control)	1	..	1.05	0.7
α-Carotene (10.5 mg/7 days)	1	0.6	0.4	1.5
α-Carotene (6 mg/3 days)	1	0.2	0.6	3.3
β-Carotene (90%β, 10%α) (7.5 mg/3 days)	2	1.6 [1]	0	10.2
Lycopene (24 mg/12 days)	4	1.2	0.4	..
Zeaxanthin (7 mg/3 days)	1	68.6 [2]	.. [2]	4.0

[1] Containing traces of α-carotene.

[2] Lutein was not separated from zeaxanthin.

TABLE 6

The presence of carotenoids in the tissues of chickens after the intravenous administration of their colloidal solutions

CAROTENOID ADMINISTERED	NUMBER OF TESTS	ADMINISTERED CAROTENOIDS IN			
		Blood plasma	Liver	Ovary	Body fat
		μg/100 ml	*μg/gm*	*μg/gm*	*μg/gm*
α-Carotene (2 mg/1 day)	1	0	4.2	0.4	0
α-Carotene (6 mg/3 days)	1	0	3.9	1.5	0.3
β-Carotene (2 mg/1 day)	1	0	5.7	1.2	0
Zeaxanthin (3 mg/1 day)	2	0	10.4	..	..

solutions. In the case of α- and β-carotenes, a similar condition also obtained after their oral administration. However, in contradistinction to the high blood levels of zeaxanthin found after this carotenoid was given by mouth, none of it could be detected in the blood after intravenous administration, although definite amounts were present in the liver. This would

seem to indicate that the carotenoids in normal blood are probably not present in colloidal form, nor are they merely in solution in blood lipids. On the other hand, it suggests that a combination of the particular blood constituent with the carotenoid takes place during the course of the absorption of this component from the small intestine. This concept is strengthened by the results of Sexton et al. ('47), who reported that β-carotene, given intravenously in fat solution, could not be used as a source of vitamin A, although large amounts were deposited in the liver. Rats dying from acute avitaminosis A had sufficient β-carotene in their livers to supply their needs for vitamin A for a number of months.

DISCUSSION

The present results confirm and extend those of earlier workers in showing that chickens can absorb the hydroxy carotenoids and store them in their tissues. The source of these carotenoids must be dietary, as is indicated by the fact that they completely disappear from the liver, ovaries and blood after the chickens have received a carotenoid-free diet over a prolonged period. However, the hydroxy carotenoids may be tenaciously retained; small amounts were present in the egg yolk of the first eggs produced by hens which had been raised on the carotenoid-free diet.

Although lutein is the principal carotenoid found in the blood, liver, ovaries and eggs of chickens on the normal farm diet, it has been demonstrated that one can expect the same situation after the administration of the dihydroxy-carotenoid, zeaxanthin, and after that of the monohydroxy-carotenoid, cryptoxanthin. The blood levels with zeaxanthin were especially high, being 184 μg % in one series and 526 μg % in a second series of tests. In the case of cryptoxanthin, the blood level was found to average 213 μg %. It would thus appear that the occurrence of these carotenoids in the blood and tissues can be considered to be adventitious.

In contradistinction to the marked effect of the carotenols in the diet on the composition of the liver and blood, the ad-

ministration of β- or α-carotene does not result in the occurrence of these provitamins A in the blood, although small amounts were found in the liver after the carotenoids were fed.

Although, in several cases, β-carotene was detected in the blood only after this carotenoid had been fed, and only a trace of α-carotene was noted following the administration of the latter compound, there were definite but minimum amounts of these carotenoids in the liver and ovaries of the chickens which were receiving supplements of these carotenoids. Moreover, the presence of β-carotene in the yolks of eggs from chickens on the farm diet was demonstrated without question. The chromogen in question must be β-carotene; this was proved by the spectrophotometric pattern in several solvents, and by mixed chromatograms of the pure carotenoids.

It has recently been shown that the chicken, in common with the rat, is able to convert β-carotene to vitamin A in the intestinal mucosa (Cheng and Deuel, '50). Ganguly, Krinsky, and Deuel ('52a) have recently demonstrated that α-carotene and cryptoxanthin are converted to vitamin A in the intestinal wall of the chicken. The latter result is supported by the demonstration that cryptoxanthin is transformed to vitamin A in the intestinal mucosa of rats (Patel, Mehl and Deuel, '51).

It would therefore seem logical to assume that, although the provitamins A can be converted to vitamin A by the intestinal mucosa of the chicken, a definite proportion may escape that fate, and be transported to the blood and ovaries. In the case of α- or β-carotene, the proportion of the provitamin A which escapes transformation to vitamin A in the intestinal wall is minimum; however, the quantity of the carotenol, cryptoxanthin, which penetrates the intestinal barrier is considerable. This opens the question of whether or not such provitamins A have an alternative site of conversion to vitamin A, in addition to the intestinal mucosa.

SUMMARY

1. It was shown that, in chickens on a farm diet, considerable amounts of lutein are present in the blood, liver, ovaries,

and in the yolks of the eggs which they produced. On the other hand, only small amounts of β-carotene were found in these biological materials.

2. The adventitious origin of the carotenoids in the tissues of chickens was proved by the fact that they could be completely removed from the tissues when the birds were maintained on a carotenoid-free diet over a prolonged period.

3. When pure cryptoxanthin or zeaxanthin was fed to carotenoid-depleted chickens, considerable amounts of the carotenoids were present in the blood, liver and ovaries. Blood levels as high as 526 μg % were noted for zeaxanthin and 213 μg % in the case of cryptoxanthin. On the other hand, when α- or β-carotene was given, only traces, if any, appeared in the blood, and minimum quantities were present in the liver, ovaries, or egg-yolks. After lycopene was fed, the average blood value was 7.4 μg %, while the amounts in the liver and in the ovaries were 1.4 and 1.2 μg per gram, respectively.

4. Proof that the small amounts of material reported as α- or β-carotene in the tissues of the chickens and in the eggs of the hens actually were these carotenoids was obtained by mixed chromatograms and by extinction maxima in several solvents.

5. When colloidal solutions of zeaxanthin, α-carotene or β-carotene were given intravenously to chickens, no trace was present in the blood after 24 hours, although considerable proportions were found in the liver. A suggested interpretation of these results is the hypothesis that carotenoids in normal blood are neither in colloidal form nor merely in solution in lipids, but instead are combined with a specific blood constituent.

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STUDIES ON CAROTENOID METABOLISM

XIII. THE CAROTENOID COMPOSITION OF THE BLOOD, LIVER AND OVARIES OF THE RAT, EWE, COW AND FROG [1, 2]

J. GANGULY, J. W. MEHL AND H. J. DEUEL, JR.
Department of Biochemistry and Nutrition, University of Southern California, Los Angeles

ONE FIGURE

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INTRODUCTION

According to the conclusions of Zechmeister ('37) and of Goodwin ('50), animals may be classified into 4 groups according to their ability to absorb and to store the several carotenoids. These include (1) those capable of storing carotene and carotenols, of which man is an example; (2) the animals storing carotenes preferentially, exemplified by the cow; (3) the group preferentially storing carotenols (chickens); and (4) those which store neither carotenes nor carotenols (pig).

In the previous paper (Ganguly et al., '53) chickens were shown to accumulate carotenols in the blood and tissues, whereas carotenes were present only in minimum amounts. The present study deals with the application of recent technics to species whose carotenoid metabolism differs from that of the fowl.

[1] Contribution 328 of the Department of Biochemistry and Nutrition, School of Medicine, and the Allan Hancock Foundation, University of Southern California.

[2] Supported by a research grant from The Nutrition Foundation.

EXPERIMENTAL

The general methods employed, and the diets, were similar to those recorded in the preceding paper of this series (Ganguly et al., '53). The rats were adult female rats of the University of Southern California strain previously fed a commercial ration.[3] The tissues and the blood samples of the cows and ewes were obtained from a local slaughter house. In each case, blood, liver and ovaries were from the same individual animal. The bull frogs *(Rana esculenta)* weighed between 200 and 700 gm, and were of both sexes. They were obtained from a local frog farm.

RESULTS

I. The presence of β-carotene in rat liver following its oral administration

In order to determine whether or not any trace of β-carotene is able to penetrate the intestinal mucosa of the rat and gain access to the blood stream and tissues, tests were carried out on 20 adult female rats. β-Carotene, dissolved in cottonseed oil, was mixed with diet 2 in such a proportion that 5 gm of the mixed diet contained 0.63 μg of β-carotene. After fasting over-night, 16 rats were given 5 gm of this diet, containing the β-carotene supplement, daily for three days. Similarly, 4 control female rats were given a daily ration of 5 gm of diet 2 without added β-carotene. All rats were sacrificed on the 5th day by heart puncture while under light nembutal anesthesia.

No carotenoid pigments could be detected in the two samples of blood or of ovarian tissue of the experimental group; the samples from 8 animals were pooled for analysis. Similarly, no chromogens were found in the pooled blood samples or in the combined livers or the combined ovaries of the control group.

On the other hand, small but definite quantities of carotene were present in the livers of the rats fed β-carotene. These livers were pooled in such a manner as to give 8 samples of

[3] Purina chow.

two livers each; they were then saponified and extracted by the usual procedure, and were chromatographed through an alumina column. The 4% acetone eluate in all groups was colored, and contained 1.5 to 2.8 μg (average, 2.1 μg) of β-carotene per gram liver.

In order to prove that the pigment isolated was, in fact, β-carotene, this fraction was transferred successively into *n*-hexane, benzene and carbon disulfide, and the extinction curves of each solution were read on the Beckman Spectrophotometer. The curves obtained on the extracts from rat livers in the several solvents, and of pure all-*trans*-β-carotene in the same solvents, are shown in figure 1.

It can be seen from figure 1 that the pigment isolated from the liver has absorption curves in the several solvents practically identical with those of pure all-*trans*-β-carotene.

A second method for establishing the nature of the liver pigment was by the use of the mixed chromatogram. A separate portion of the 4% acetone fraction was taken up in light petroleum ether (b.p., 60 to 70°C.) and was chromatographed through a magnesia : celite (1 : 1) column with added all-*trans*-β-carotene. No separation of pigment into separate bands could be observed. This establishes the identity of the two mixed pigments, one of which was known to be pure all-*trans*-β-carotene. On the basis of the identical absorption spectrum of the liver pigment and of pure all-*trans*-β-carotene in several solvents, and of the fact that they give a single chromatogram when mixed, there would seem to be no doubt that the pigment present in the rat liver under these circumstances is β-carotene.

II. The pigment distribution in ewes, cows and bull frogs

The distribution of carotenes and xanthophylls (chiefly lutein) in the blood, livers and ovaries of the several types of animals is recorded in table 1.

Vitamin A, as determined by the Carr-Price reaction at 620 mμ, was detected in variable amounts in the livers, kid-

neys and fat bodies of frogs and tadpoles. None could be detected in the ovaries of frogs or cows.

Although no information is available on the diet of the cows and ewes prior to slaughtering, it seems probable that they had been receiving appreciable amounts of carotenoids in

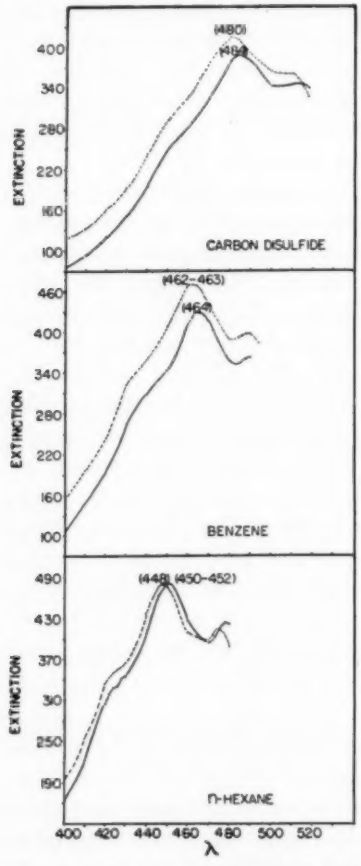

Fig. 1 The absorption curves of all-*trans*-β-carotene (solid lines) and of pooled samples of the chromogen of rat liver (dashed lines) in *n*-hexane, carbon disulfide and benzene. The figures on the curves represent the $\lambda_{max.}$ for each solvent.

TABLE 1

The distribution of carotenoids in the tissues of several species on a normal diet

GROUP AND SPECIES	NUMBER OF TESTS	CAROTENES [1]			XANTHOPHYLLS [1]		
		Plasma	Liver	Ovaries	Plasma	Liver	Ovaries
		μg/100 ml	*μg/gm*	*μg/gm*	*μg/100 ml*	*μg/gm*	*μg/gm*
Group 1 (Low carotene, low carotenol)							
Ewe	3	0	0.5 (0.3–0.6)	0	0	0.4 (0.3–0.4)	0
Rat (Carotene feeding)	8	0	2.1 (1.5–2.8)	0	0	0	0
Rat (Carotene-free diet)	4	0	0	0	0	0	0
Group 2 (Low carotene, high carotenol)							
Chicken [2]	4	4.2 (6.3–0)	1.3 (0.5–2.7)	0.8 (0.7–1.0)	162 (70–231)	7.6 (3.4–12.5)	35.1 (14.3–51.0)
Group 3 (High carotene, low carotenol)							
Cow	4	435 (340–500)	20.6 (12.0–32.6)	112.8 (8.5–285)	16.9 (5.8–45.0)	1.2 (0.8–1.3)	3.1 (0.9–7.2)
Group 4 (High carotene, high carotenol)							
Frog, male	3	0	14.7 (4.9–24.6)	..	..	7.7 (2.2–16.7)	..
Frog, female	5	0	8.5 (5.9–9.1)	3.9 (1.2–9.3)	..	3.7 (2.2–6.4)	10.5 (4.4–14.8)

[1] Figures in parentheses are the range of values.
[2] Results of Ganguly et al. ('53).

the diet, inasmuch as they are both herbivora and, in all probability, would have received a diet containing alfalfa.

In the case of the frogs, an unsuccessful attempt was made to deplete them of carotenoids by fasting them for three to 4 weeks. No appreciable change in carotenoids occurred in the tissues as a result of this treatment. Experiments were carried out with giant tadpoles, which were kept in a small aerated tank and were maintained on a carotenoid-free diet (diet 2 described by Ganguly et al., '53). The diet was sprinkled on the water and was apparently consumed, judging by the copious amounts of excreta which collected in the tank. It was possible to maintain the tadpoles for several weeks by changing the water daily and adding fresh diet. However, no appreciable changes in carotenoid concentrations in the tissues could be observed, even after an interval of several weeks.

DISCUSSION

The experiments described here, together with those on chickens reported earlier (Ganguly et al., '53), confirm the fact that animals fall in at least 4 groups insofar as their ability to absorb and deposit different carotenoids is concerned. In group 1 (low carotene, low carotenoid), the only carotenoids reported are in the liver, while in group 4 they occur both in the liver and in the ovaries. However, in neither of these groups could any appreciable quantity of the carotenoids be detected in the blood.

In contradistinction to this result, the level of carotenols was extremely high in the blood of the chickens (group 2), and the amount in the ovaries exceeded that in the liver. Likewise, in the case of group 3 (high carotene, low carotenol), the blood level of carotene far outstripped that of carotenoids in the blood of other species.

The main site of deposition of carotenoids, in types of animals capable of absorbing these pigments (groups 2, 3 and 4), was shown to be in the reproductive organs.

The question which naturally arises is why such wide variations obtain between the different animals as regards their

relative ability to absorb the several types of carotenoids. A similar related question which should be considered is why vitamin A is deposited in the liver and not in the body fat. Also, it is pertinent to inquire why the concentrations of carotenoids are so low in the liver. Another unanswered problem is why a high concentration of β-carotene and no vitamin A occurs in the cow's ovaries, as contrasted with the high concentrations of xanthophylls and smaller amounts of vitamin A which are present in hen's ovaries and egg yolks (Ganguly et al., '53).

In addition to our own work, many other workers have earlier cited similar examples of the selective absorption of the carotenoids. For example, Zechmeister and Tuzson ('35) have recorded that β-carotene preponderates in the tissues of the horse, although the proportion of xanthophylls far exceeds that of β-carotene in the diet. These carotenols have likewise been isolated from the intestine of the horse, and have also been shown to be present in the feces (Zechmeister and Tuzson, '34). By analysis of blood samples obtained from the portal and jugular veins of a horse after a high carotenoid diet, these workers were unable to find any trace of xanthophylls. Their obvious conclusion was that the selection takes place in the intestine.

More recently, the experiments of Goodwin et al. ('46) and of Thompson et al. ('50) have clearly demonstrated that, in the goat and in the pig, carotenoids do not appear in the blood from the portal or jugular veins, irrespective of the diet. This fact likewise indicates that the site of the selection (or of the rejection) of the carotenoids is the intestine.

The variations in absorption of the carotenoids can probably not be ascribed to a preferential destruction. This explanation for the failure of xanthophylls to appear in the blood or tissues of the horse is ruled out by Zechmeister and Tuzson ('35). The experiments of Goodwin et al. ('46) and Thompson et al. ('50) similarly exclude the concept that all carotenoids are destroyed in the body as an explanation for their failure to appear in the blood. However, if one con-

siders the change of provitamins A to vitamin A in the intestinal wall as an example of carotenoid destruction, it is possible that the variations between group 1 and group 3 might be ascribed to a similar conversion. However, there is no evidence that an effect of this nature may apply to carotenols.

One possible explanation for this divergent behavior in carotenoid composition between the different groups of animals is that specific receptors for vitamin A and for the carotenoids are present in the different tissues. We assume that these receptors are specific proteins. Here again, the extent of the capacity of the protein to bind the carotenoid would determine the amount absorbed. Likewise, the presence in the cow's ovary of a specific protein receptor for β-carotene would explain the high concentration of β-carotene in this tissue. Moreover, the presence of another receptor protein for vitamin A ester in the liver would clarify the relative abundance of the ester in this tissue.

Although direct evidence for such a concept is not available, there is considerable indirect proof in its favor. Palmer ('22) isolated a carotene-protein complex from cow's blood, and postulated the existence of a carotene-albumin complex. It was demonstrated by Dzialoszynski et al. ('45) that carotenoids and vitamin A are associated with proteins in human blood; moreover, Mehl ('42) observed that the carotenoids in human blood are largely concentrated in a fraction rich in β-globulin. More recently, Oncley et al. ('50) have noted that the carotenoids in human blood occur almost exclusively in the β-lipoprotein fraction. Finally, Ganguly and associates ('52) have presented evidence that vitamin A ester, vitamin A alcohol and carotenoids are associated with different plasma proteins in several species of animals.

In addition to the evidence of carotenoid-protein complexes in the blood, there are some reports which indicate that the same principle applies to carotenoid and vitamin A deposition in the liver. Baumann and co-workers ('42) and Abels

et al. ('42) previously suggested the concept of protein anchorage of vitamin A in the liver.

Bukin and Areshkina ('40) reported that 63% of the fat may be obtained by centrifugation or direct ether extraction of cod livers, but only 25% of the vitamin A. The remaining 75% of the vitamin A and 37% of the fat can be extracted with ether after denaturation of the proteins. These same authors report that all of the provitamin and vitamin D and sterols are associated with soluble proteins in the liver of fish and molluscs. More recently, using the technic of differential centrifugation, Krinsky and Ganguly ('53) have observed that, in rat liver homogenates, vitamin A ester and vitamin A alcohol are associated with different cell fractions. In homogenates of beef liver, β-carotene, vitamin A ester and vitamin A alcohol, respectively, are each chiefly associated with a different cell fraction (Krinsky and Ganguly, '52).

Protein-carotenoid complexes have been reported in tissues other than the liver. Stern and Salomon ('38) isolated an astacene-protein complex from lobster eggs, which they called ovoverdin. Moreover, the vitamin A aldehyde-protein combination in the retina is another example. The extreme specificity of this coupling has recently been demonstrated by Hubbard and Wald ('52a, b). It was reported that only certain stereoisomeric forms of vitamin A would combine with the protein fraction to yield rhodopsin.

SUMMARY

1. The carotenoid present in minimum quantities in the liver of the rat after the administration of β-carotene has been shown by unequivocal methods to be all-*trans*-β-carotene.

2. Only traces of carotene and of carotenols were present in the liver of the ewe. No carotenoids were found in the blood or ovaries.

3. On the other hand, large quantities of β-carotene were present in the blood, liver and ovaries of the cow, while small but definite amounts of carotenols were likewise present. The carotene content of the ovaries far exceeded that of the liver.

4. In the case of frogs, definite amounts of β-carotene and carotenols occurred in both the liver and the ovaries. However, no carotene could be detected in the blood.

5. It is suggested that the variation in absorption and deposition of the carotenoids in the tissues as related to the species of the animal may be related to the presence or absence of appropriate protein receptors in the blood and tissues of these animals. Numerous examples of such carotenoid and vitamin A combinations with protein in the blood and tissues are cited in support of this concept.

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COMPARISON OF CHEMICAL ANALYSIS AND BIOASSAY AS MEASURES OF VITAMIN A VALUE: YELLOW CORN MEAL

ELIZABETH CROFTS CALLISON, LOIS F. HALLMAN,[1] WILLIAM F. MARTIN[2] AND ELSA ORENT-KEILES[2]

WITH THE TECHNICAL ASSISTANCE OF EMILY S. CONWAY AND ELSIE CRUMP

Bureau of Human Nutrition and Home Economics, Agricultural Research Administration, U. S. Department of Agriculture, Washington, D. C.

ONE FIGURE

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The nature of the vegetable or fruit has been shown to influence the availability of its carotenoids to the animal body to such a marked degree as to render their carotene content unreliable as an index of the biological vitamin A value of these foods. Therefore it is necessary to differentiate "chemical" from "biological" vitamin A units in tables of food composition, and to establish factors for the various plant foods by means of which one type of unit can be converted to the other in making dietary calculations. Parallel quantitative measurements of carotene content and vitamin A value of strictly comparable samples of carrots, sweet potatoes and kale (Callison et al., '49) have been made in this laboratory. Yellow corn meal has now been assayed by a similar procedure.

Although corn makes up only about one-tenth of the cereal eaten in the United States, it ranks second to wheat in per

[1] Present address: National Institutes of Health, U. S. Public Health Service, Bethesda, Maryland.

[2] Present address: Division of Research Grants, National Institutes of Health, U. S. Public Health Service, Bethesda, Maryland.

capita consumption (Bureau of Agricultural Economics, '49) and is heavily relied upon as a dietary staple when income is low (Bureau of Human Nutrition and Home Economics, '49). It either rivals or surpasses wheat and rice as the chief dietary cereal in a number of countries, for example, Yugoslavia, South Africa, Egypt, Brazil, Columbia, Peru, and countries of Central America (Food and Agriculture Organization of the United Nations, '49), and may constitute the chief article of diet for some population groups. White corn meal is used predominantly in the United States (Bureau of Human Nutrition and Home Economics, '49), but yellow corn meal is the favored variety in other parts of the world, and its use is encouraged there by nutritionists [3] because the carotene content of yellow corn, although small in comparison with that of many green and yellow vegetables, is of crucial importance to the vitamin A supply of these people.

Corn is the only cereal that contains significant amounts of vitamin A-active carotenes, and is unique among common plant foods in that cryptoxanthin rather than β-carotene is responsible for most of its vitamin A activity.

MATERIALS AND METHODS

Sufficient freshly ground yellow corn meal for the whole experiment was procured at one time from a mill in Washington, D. C., and stored unopened in heavy paper-bag containers at 5°C. The corn meal was old process water-ground from the whole yellow corn of hybrid varieties, grown in Maryland and Virginia. The laborious procedures of cooking the corn meal as for human food, and then drying the product for bioassay, were omitted, since extensive experiments in this laboratory (Hewston et al., '48) had demonstrated that the biological vitamin A value of yellow corn meal was unchanged by two hours of cooking over boiling water.

Since corn has been reported (Fraps and Kemmerer, '41) to show considerable varietal differences, both in the com-

[3] Personal communication, Charlotte S. Chatfield, Food and Agriculture Organization of the United Nations, Rome, Italy.

ponents of the carotenoid pigments and in their proportionate amounts, chromatographic studies were made both to identify the individual pigments and to measure quantitatively those possessing provitamin A activity.

Identification and measurement of carotenoids

Preliminary chromatographic separation of the corn carotenoids was made on MgO Hy-flo Supercel; the analyses were carried out essentially according to procedures described in "Methods of Vitamin Assay" (Association of Vitamin Chemists, '47). A 25-gm sample of corn meal was saponified in a Waring Blendor for 5 minutes with 100 ml of 12% methanolic KOH at room temperature, filtered through a fritted glass funnel, and the residue extracted once with a mixture of equal parts of acetone, methanol and petroleum ether (purified Skelly-solve fraction B, b.p. 60–70°C.), followed by successive portions of acetone until it was colorless. The several extracts were combined in a separatory funnel, the carotenoid pigments transferred to the petroleum ether layer by the addition of H_2O with cautious agitation, and the acetone-water layer drawn off. Complete removal of carotenoids from the acetone-water phase was effected by successive extractions with petroleum ether. The petroleum ether extracts were then combined, washed free of acetone and alkali with H_2O, dried over anhydrous Na_2SO_4, and concentrated *in vacuo* to a 5- to 10-ml volume. The concentrate was then chromatographed on an ice water-cooled 1:3 column of MgO [4] Hy-flo Supercel and developed with petroleum ether (Skelly-solve, fraction B). When the bands had separated fully, they were eluted with 1% acetone-petroleum ether solution for the first bands off the column, and 30% acetone for the more strongly adsorbed bands at the top. The several bands were collected individually, evaporated to dryness *in vacuo* and the residue taken up in petroleum ether for spectrophotometric measure-

[4] Magnesium oxide powder, "Baker's Analyzed" reagent.

ments. A complete absorption curve of each pigment fraction was made with the Beckman DU model spectrophotometer, and this curve, together with the position of the band on the column, was used in attempting to identify the various carotenoids.

Since the biologically important β-carotene fraction gave evidence of the presence of isomers in considerable amounts, another chromatographic procedure was employed for their separation. The carotenoids from 50 gm of corn meal, saponified and extracted as described previously, were passed through a column of confectioner's XXXX sugar; the carotenes and cryptoxanthin passed into the effluent, while the xanthophylls other than cryptoxanthin were adsorbed on the sugar. Cryptoxanthin was removed from the effluent with 92% methanol, three successive extractions usually being sufficient for complete removal; the petroleum ether fraction was then washed with H_2O, dried over anhydrous Na_2SO_4, evaporated to small volume, and diluted to 5 ml with petroleum ether containing 0.5% acetone. One milliliter of the solution was reserved for spectrophotometric readings of the unchromatographed pigments and the remaining 4 ml were passed through an ice water-cooled column of hydrated lime.[5] The column was developed and the first three fractions (a, b, c) eluted with 0.5% acetone-petroleum ether, the 4th fraction (d) with 5% acetone. Each fraction was collected separately and rechromatographed on lime from one to 5 times, in order to encourage further possible separations.

Finally, each fraction was evaporated to dryness, diluted to a volume suitable for reading in the spectrophotometer (10 ml for a, b, c, and 25 ml for d), and absorptions in the range of 360 to 500 mμ were measured. Fractions c and d were tested with concentrated HCl, and fraction d with iodine in an attempt to identify and further characterize these pigments.

[5] Shell brand, conditioned over saturated NaCl solution for 48 hours.

Preparation of corn meal and corn meal extract for bioassay

For the biological studies, 7,000 gm of the corn meal were thoroughly mixed by hand, and two 2,500-gm portions removed for preparation of an extract containing the total carotenoid fraction for bioassay. The remaining 2,000 gm were reserved for bioassay of the whole cereal. In preparing the extract, 2,500 gm of the meal were placed in a large percolator and methanol, acetone and diethyl ether successively allowed to flow by gravity through the material. Each solvent was used for approximately 25 hours; at the end of this time, the cereal was white and 100 ml of solvent collected at the outlet were colorless. The extract was concentrated *in vacuo* to approximately 1 l, and saponified by allowing to stand at room temperature for 65 hours with 500 ml of 20% methanolic KOH and 1,000 ml diethyl ether, with occasional stirring. The pigments were transferred to diethyl ether by the addition of H_2O, and the aqueous phase was drawn off and re-extracted several times with ether. All ether extracts were combined, washed with H_2O, dried over Na_2SO_4, and concentrated to approximately 50 ml *in vacuo*. The extract was transferred to a weighed flask, using small amounts of refined cottonseed oil [6]; the remaining ether was removed by subjecting the extract to a vacuum until the weight remained constant, which required approximately 20 hours. The concentrated oil solution was stored at 0°C. and diluted, as needed, with cottonseed oil so that 0.1 ml of the concentrate represented either 1 or 2 gm of corn meal, for feeding at two levels of vitamin A supplementation.

Biological assay

In the interests of greater specificity and precision, the laboratory procedures for vitamin A bioassay employed in previous studies (Callison et al., '49) have been modified both as to the basal diet and the preparation of animals for

[6] Wesson oil.

the assay. Experiment [7] has demonstrated that the casein employed in the vitamin A assay diet in this laboratory was deficient in vitamin B_{12}, as had been suggested by Hartman et al. ('41, '49), and that when used in the assay of a food containing both vitamins, an erroneously high vitamin A value was indicated. In accordance with these observations, supplementary liver extract was fed in conjunction with the assay diet which contained: 18% alcohol-extracted casein, 10% dried brewers' yeast, 4% Osborne-Mendel salt mixture, 5% refined cottonseed oil (containing 0.1% alpha-tocopherol), 63% cornstarch, and 0.3 ml viosterol per kilogram of diet.

Pregnant rats were maintained on the laboratory stock ration until the 16th day of pregnancy, as measured from the appearance of the placental sign, when the vitamin A-free diet was given them, together with 0.2 ml liver extract,[8] three times a week, and continued through lactation. The young were weaned at 18 to 20 days, when they weighed 30 to 39 gm, and the males placed on the assay diet and fed by tuberculin syringe 0.1 ml liver extract three times a week. When weight either remained stationary or declined over a period of 5 days, ophthalmia was usually present, and supplementation with vitamin A was begun. Four rats from the same litter were given the assay material at two levels; as a reference standard, β-carotene [9] was given in cottonseed oil at two levels. In addition, a group of rats, usually one from each litter, was kept without vitamin A supplementation as negative controls. Individual animals were assigned to the various supplements on the basis of the order of depletion, as suggested by Bliss (György, '51). Twelve litters (a total of 48 animals) were employed in each of the two assays, i.e., corn meal and corn meal extract; data from the whole litter were discarded when one or more animals died before completion of the assay, and a new litter was substituted. No missing values were replaced by calculation.

[7] Unpublished data, Bureau of Human Nutrition and Home Economics.

[8] Lederle's liver extract for injection, 15 units per milliliter.

[9] Pure β-carotene, General Biochemicals.

Supplements were fed thrice weekly, and were stored at 0°C. between feedings. The corn meal was weighed on a semi-analytical balance into a small earthenware cup, and the animal was allowed to eat it voluntarily. The oil solution of β-carotene or of corn meal extract was fed by tuberculin syringe. Fresh feeding solutions were prepared from the stock solution once a week. Assays were continued for 4 weeks. The method of calculating the potency of the unknown and its standard error was that described by Bliss (György, '51).

RESULTS AND CONCLUSIONS

Chemical analyses

The carotenoid pigments of the corn meal studied in these experiments separated into 7 bands on 1 : 3 MgO Hy-flo Supercel. The first band eluted was faint orange, and from its position on the column was tentatively identified as α-carotene; the second was deep orange and apparently contained the all-trans-β-carotene, although the ratios of absorptions at 430, 450, and 480 mμ gave evidence of the presence of isomers in large amounts; the third band was canary yellow and its absorption spectrum was strongly suggestive of K-carotene, first described by Fraps and Kemmerer ('41) in yellow corn; the 4th band was cryptoxanthin, probably containing traces of neo-cryptoxanthin, since a faint zone was distinguishable below the main band but could not be separated from it. Accuracy in the calculation of "chemical" vitamin A units would be slightly if at all influenced by combining the cryptoxanthin isomers; therefore no further effort was made to separate them. Lutein and zeaxanthin were identified in the 5th and 6th bands, respectively, and the 7th, which clung tenaciously to the top of the column, was classed as "impurities."

Quantitative measurement of the cryptoxanthin in 6 25-gm samples of the corn meal averaged 2.4 μg per gram of corn meal. Because gross errors in the measurement of vitamin

A potency can result when any considerable amount of partially or wholly inactive pigments are calculated as all-trans-β-carotene, resolution of the carotene fraction was undertaken, as described. Four fractions, a, b, c, and d, separated during the initial chromatographing on hydrated lime. Fraction a, the first to be eluted, had maxima at 445 and 472 mμ, with a shelf at 420 mμ; since both the absorption maxima and the ratios between them were very similar to those reported for neo-β-carotene B by Bickoff and his associates ('48), fraction a was identified as this isomer of β-carotene. No zone with the absorption characteristics of α-carotene appeared on the column.

Upon rechromatographing fraction b on lime, it separated into two components, b_1 and b_2; b_1, with maxima at 478 and 450 mμ, was identified as reasonably pure all-trans-β-carotene; fraction b_2 had maxima at 448 and 475 mμ and was identified as neo-β-carotene U (Bickoff et al., '48).

The absorption curve of fraction c (fig. 1) proved to be identical with that determined for ζ-carotene isolated from tomatoes and carrots by Nash and Zscheile ('45), and found in Katahdin potatoes by Brunstetter and Wiseman ('47), except for a minor absorption peak at 450 mμ which appeared in all preparations of fraction c, even after 5 rechromatographings on lime, and which is completely lacking from the ζ-carotene curve. Nash and Zscheile ('45) believed that a similar pigment ("unnamed carotene 1") separated from yellow corn by White, Zscheile and Brunson ('42) was predominantly ζ-carotene contaminated with a small amount of another carotenoid. However, there is evidence from several sources that we may be dealing with a carotenoid distinct from ζ-carotene. Baumgarten, Bauernfeind, and Boruff ('44) have also described such a pigment occurring in corn and corn distillers' by-products. Recently Goodwin ('52) reported the occurrence in *Phycomyces Blakesleeanus* of a pigment having all the properties of ζ-carotene except that "it always showed a small additional absorption band at 451 mμ," which "does not appear to be due to an impurity,

for repeated chromatography on different adsorbents not only failed to eliminate it but did not alter its E value relative to those of the main bands." Bauernfeind et al. ('44) produced a pigment with absorption maxima qualitatively similar but differing quantitatively, by chromatographing commercial preparations of β-carotene on activated lime and developing

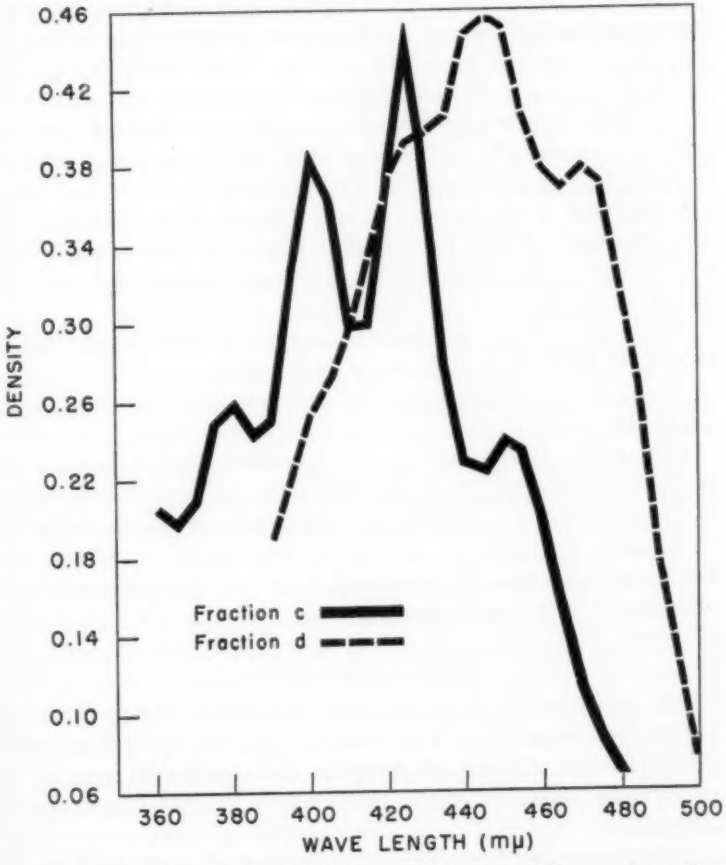

Fig. 1 Absorption spectra of fractions c and d separated from crude carotene of yellow corn meal by means of $Ca(OH)_2$.

with unpurified chloroform, and suggested that their pigment was an intermediate step in the transformation of β-carotene to the ζ-carotene-like pigment in corn.

The absorption spectrum of fraction d (fig. 1) had maxima at 470.5 and 446 mμ and was similar to carotenoid X obtained by Kemmerer and Fraps ('43) from dehydrated alfalfa, not only in its absorption spectrum but also its position on the $Ca(OH)_2$ adsorption column and its great stability, having remained unchanged for as long as a month when stored in petroleum ether at about 5°C. in the dark. Since Le Rosen and Zechmeister ('42) had reported maxima at 470 and 443.5 mμ as well as other characteristics similar to those of fraction d for prolycopene from the tangerine tomato, and since a relationship had been shown between prolycopene and ζ-carotene (Porter and Zscheile, '46; Nash, Quackenbush and Porter, '48), fraction d was tested with iodine in petroleum ether for the rapid isomerization characteristic of prolycopene, with negative results.

Neither fraction c nor d developed a blue color when shaken with 25% HCl, thus indicating that neither was a diepoxide of β-carotene, as described by Karrer and his co-workers ('45, '48, '50).

Systematic investigation of the carotenoid pigments of *Zea mays,* of which there are numerous red and yellow varieties, could well be as fruitful in elucidation of the mechanisms of carotene biosyntheses as similar investigations of the pigments of tomatoes (Porter and Lincoln, '50; Goodwin and Jamikorn, '52) have been.

Vitamin A activity

The provitamin A carotenoids present in this sample of yellow corn meal were 4 in number; i.e., cryptoxanthin, neo-β-carotene B, all-trans-β-carotene, and neo-β-carotene U, in decreasing order of their contribution to total vitamin A activity.

ζ-Carotene has been shown (Porter et al., '46) to have no biological activity, therefore no activity was ascribed to the

pigment in fraction c in this investigation. Since in this sample of corn there was at least twice as much of this pigment as of β-carotene, it could cause serious inaccuracies in estimating vitamin A value by applying a correction factor to a crude carotene determination.

Kemmerer and Fraps ('43) showed carotenoid X to be devoid of biological activity, and therefore fraction d in the present study was regarded as being without vitamin A value. If undetected in the β-carotene fraction, this carotenoid would

TABLE 1

Contribution of provitamin A pigments of yellow corn meal to its "chemical" vitamin A value

CAROTENOID	CONTENT[1]	VITAMIN A VALUE[2]
	μg/100 gm	*I.U./100 gm*
All-trans-β-carotene	31.1	51.8
Neo-β-carotene B	39.6	35.0
Neo-β-carotene U	21.5	13.6
Cryptoxanthin	236.6	216.9
	Total	317.3

[1] Specific absorption coefficients at wave length 450 mμ, as determined by Bickoff et al. ('48), used in calculations.

[2] Six-tenths microgram of all-trans-β-carotene is equivalent to one international unit; neo-β-carotene B is considered to have 53%, neo-β-carotene U 38% and cryptoxanthin 55%, of the vitamin A value of all-trans-β-carotene.

have constituted a serious source of error in the estimation of vitamin A potency, since it accounted for more than three-fourths of the absorption at 450 mμ and only one-fourth was due to all-trans-β-carotene. Such an error could easily occur, since it is not separated from β-carotene on MgO Hy-flo Supercel.

Vitamin A value as estimated from the amounts of the various provitamin A pigments present in this specimen of yellow corn meal is summarized in table 1, and totalled 317 international units. In the calculations, 0.6 μg all-trans-β-carotene is considered to equal one international unit; cryp-

TABLE 2

Summary of bioassay data used in calculation of vitamin A value of yellow corn meal and of its extracted carotenoids

WEEKLY DOSAGE LEVELS	NO. RATS	WEANING		DEPLETION		AVE. WT. GAINED DURING ASSAY PERIOD	VITAMIN A VALUE OF CORN MEAL
		Age	Wt.	Time required	Wt. gained [1]		
		days	*gm*	*days*	*gm*	*gm*	*I.U./100 gm*
Assay I							
R_1 [2] 6.3 μg β-carotene	12 M	19.0 ± [3] 0.23	36.8 ± [3] 4.05	38.4 ± [3] 4.62	138.0 ± [3] 24.58	11.5 ± [3] 4.79	
A_1 3 gm corn meal	12 M	19.0 ± 0.23	36.9 ± 3.58	38.2 ± 5.21	132.3 ± 21.52	9.1 ± 3.88	
R_2 12.6 μg β-carotene	12 M	19.0 ± 0.23	36.9 ± 2.81	37.8 ± 4.86	140.2 ± 26.22	24.8 ± 4.50	
A_2 6 gm corn meal	12 M	19.0 ± 0.23	35.6 ± 3.12	38.4 ± 4.78	127.9 ± 22.27	18.0 ± 4.92	264 [4] 7.5%
Assay II							
R_1 6.3 μg β-carotene	12 M	19.1 ± 1.32	36.5 ± 3.20	35.5 ± 4.87	107.5 ± 27.57	12.0 ± 2.58	
A_1 extract from 3 gm corn meal	12 M	19.1 ± 1.32	36.2 ± 4.07	36.4 ± 4.94	110.0 ± 28.59	10.4 ± 3.45	
R_2 12.6 μg β-carotene	12 M	19.1 ± 1.32	35.8 ± 3.22	34.3 ± 4.54	104.8 ± 15.41	20.0 ± 5.38	
A_2 extract from 6 gm corn meal	12 M	19.1 ± 1.32	36.1 ± 4.19	34.7 ± 4.38	106.2 ± 24.57	19.7 ± 6.41	324 [4] 8.7%

[1] Liver extract was responsible for large weight gains during depletion; in spite of this, growth responses to vitamin A supplementation were considerable and were proportional to dose of vitamin A.

[2] R_1 and R_2 represent the low and high levels of the carotene fed as a standard of reference; A_1 and A_2 represent similar levels of the substance under assay.

[3] Standard deviation.

[4] Per cent standard error.

toxanthin is taken to have 55% of the potency of all-trans-β-carotene (Deuel et al., '45a), neo-β-carotene B 53% (Deuel et al., '45b) and neo-β-carotene U 38% (Deuel et al., '44) of that potency.

Bioassay data for the vitamin A value both of corn meal and of corn meal extract are presented in table 2. The vitamin A value of the corn meal was 264 I.U. per 100 gm, whereas the extract of 100 gm of the meal had a value of 324 I.U. The biological vitamin A value of the corn meal extract agreed within the limits of experimental error with that anticipated from chemical analysis of the corn meal; i.e., 324 and 317 I.U. per 100 gm, respectively. Similarly, in previous studies with other vegetables (Callison et al., '49), the carotenes in the vegetable extracts always proved to be 100% available, regardless of availability from the vegetable itself. The possibility was kept in mind that a small amount of all-trans-β-carotene might have changed to less active isomers during the months of storage required satisfactorily to separate and identify the various pigments, despite the fact that the corn meal was kept in the dark at about 5°C. However, in view of the agreement between the "chemical" vitamin A value of the meal and the "biological" value of the extract, such losses must have been minimal.

The availability to the rat of the biologically active carotenes of yellow corn meal appears, from this study, to be high; i.e., 82%. In contrast, Wilson, Ambrose, DeEds, Dutton and Bailey ('46) have reported the availability of carotene from corn to be poor; i.e., only about 52%. If, in the present investigation, the calculations had been made on the basis of pigments separated on MgO Hy-flo Supercel, the corn meal would have had an apparent "chemical" vitamin A value of 502 I.U. per 100 gm, and availability would have been only about 53%. It seems probable, therefore, that the analytical procedures employed by Wilson et al. failed to remove some inactive pigment from the β-carotene, and thus led to exaggerated estimates of "chemical" vitamin A content.

SUMMARY

The provitamin A carotenoids of yellow old process water-ground corn meal were separated by chromatographic techniques and measured spectrophotometrically. The vitamin A values of the corn meal and of its carotenoid extract were determined by rat growth bioassay, and availability of the carotene calculated.

The "β-carotene" fraction was found to include significant amounts of the isomers neo-β-carotene B, neo-β-carotene U, and a carotenoid which may well be identical with a pigment isolated from dehydrated alfalfa by other investigators, but which, to our knowledge, has not been reported previously in corn. It was necessary to chromatograph the β-carotene fraction on $Ca(OH)_2$ in order to distinguish between these isomers.

Another pigment, which may be either "contaminated" ζ-carotene or another carotene closely related to it, occurred in considerable quantities, and was separated from β-carotene both by MgO Hy-flo Supercel and by $Ca(OH)_2$. These last two pigments were compared with similar carotenoids which have been described by other investigators, and some attempt was made to characterize them further.

It was found that the bioassay vitamin A value of the corn meal extract, 324 I.U. per 100 gm meal, agreed with the vitamin A potency calculated from chemical analysis, 317 I.U. per 100 gm meal, when hydrated lime was employed as adsorbent to separate an inactive pigment and the partially active isomers of β-carotene from all-trans-β-carotene, all of which compounds remain together on another common adsorbent, MgO Hy-flo Supercel. The biologically active carotenes, as they occur in corn meal, proved to have a biological vitamin A value of 264 I.U. per 100 gm and therefore to be 82% available to the rat. It is suggested that the poor availability reported by other investigators may be accounted for by the inclusion of considerable amounts of inactive pigments with the β-carotene fraction.

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STUDIES ON THE REVERSAL OF AMINOPTERIN TOXICITY IN THE RAT WITH CITROVORUM FACTOR, FOLACIN AND RELATED COMPOUNDS [1]

H. E. SAUBERLICH

Department of Animal Husbandry and Nutrition, Agricultural Experiment Station of the Alabama Polytechnic Institute, Auburn

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In 1948 Oleson, Hutchings and Subbarow reported that 4-aminopteroylglutamic acid (aminopterin) was a potent antagonist of pteroylglutamic acid (folacin) for *Streptococcus faecalis* R, rats and chickens. They observed that the inhibition was reversed only with great difficulty with very high amounts of folacin. Similar observations were made by Higgins ('49) with rats and by Franklin et al. ('48) with mice. More recently it was noted that the factor required for the growth of *Leuconostoc citrovorum* (Sauberlich and Baumann, '48) was very effective in reversing aminopterin inhibition in this organism (Sauberlich, '49a) and in *S. faecalis* (Sauberlich, '52b; Broquist et al., '50). Nichol and Welch ('50a) observed that concentrates of the citrovorum factor were effective against aminopterin toxicity in the rat. Broquist et al. ('50) also observed that concentrates of the principle were effective against aminopterin toxicity in the mouse. Recently a synthetic compound, leucovorin, possessing activity identi-

[1] Published with the approval of the Director, Agricultural Experiment Station, Alabama Polytechnic Institute. The aureomycin, folacin, aminopterin and synthetic citrovorum factor (leucovorin) were generously furnished by the Lederle Laboratories Division, American Cyanamid Company. The cortisone acetate and all vitamins were supplied by Merck and Company, Inc. A preliminary report of this investigation was presented before the Southeastern Regional Meeting of the American Chemical Society, Auburn, Alabama, October, 1952.

cal or very similar to that of the natural citrovorum factor (Broquist et al., '51, '52; Brockman et al., '50; Sauberlich, '52b) has been found to be very effective in reversing aminopterin toxicity in bacteria (Sauberlich, '52b; Broquist et al., '52) and in mice (Broquist et al., '52).

When the synthetic citrovorum factor (leucovorin) became available, experiments were undertaken in this laboratory to study the effectiveness of this and related compounds in preventing aminopterin toxicity in the rat. Some of the results of these experiments are reported in the present paper.

EXPERIMENTAL

Weanling rats of either the Alabama Experiment Station strain (AES, mixed-sex) or Sprague-Dawley strain (SD, male) were placed individually in wire-bottomed cages. Food and water were given ad libitum and the animals were weighed at weekly intervals. All experiments were repeated one or more times. The animals received the basal diet or the indicated modifications of it. The basal diet had the following percentage composition: methanol-extracted casein 20, corn oil 4, cod liver oil 1, sucrose 70.5, salts [2] 4, L-cystine 0.3, choline chloride 0.2. The following vitamins were added, in milligrams per kilogram of diet: α-tocopherol 25, α-tocopherol acetate 25, 2-methyl-1, 4-naphthoquinone 5, inositol 1,000, niacin 20, calcium pantothenate 30, thiamine 6, riboflavin 6, pyridoxine 6, folacin 1, biotin 0.5, and vitamin B_{12} 0.03.

The modifications in the diet consisted of supplementations of aminopterin, folacin, synthetic citrovorum factor (CF), penicillin, aureomycin or protein. Certain groups of rats received daily intraperitoneal injections of folacin, CF, vitamin B_{12}, ascorbic acid or cortisone acetate. Blood studies were made routinely on all animals, and tissues were examined grossly for pathological changes. In several groups of animals, ascorbic acid determinations were made as well as studies on the *in vitro* formation of methionine from DL-homocysteine.

[2] Salmon, W. D., J. Nutrition, *33:* 155, 1947.

RESULTS

Effectiveness of injected or dietary folacin and CF on aminopterin toxicity in the rat

Typical results with respect to the toxicity of aminopterin for the rat are shown in table 1 (groups 1–3). A level of 0.75 to 1.0 mg of aminopterin per kilogram of diet was very toxic to weanling rats. At the higher level of aminopterin, the majority of the animals died within 10 to 20 days after being placed on experiment. Growth of the animals was slow, followed by weight loss and death. Aminopterin fed at a level of 0.75 mg per kilogram of diet was completely counteracted by daily intraperitoneal injections of 40 μg of CF, whereas similar injections of folacin were only partially effective. When the aminopterin was fed at the 1-mg level, injections of 100 μg per day of CF were required for full protection, although injections of only 25 μg afforded partial protection (table 1, groups 6–8). However, at this level of aminopterin in the diet, folacin was essentially ineffective, even with daily injections of 250 μg (table 1, groups 9–12). Daily injections of 10 μg of vitamin B_{12} were ineffective. Rats fed the basal diet or rats completely protected against the effects of aminopterin consumed 10 to 11 gm of food daily. Thus, the rats ingested 7.5 to 11 μg of aminopterin daily (table 1, groups 4 and 8).

Because of the acid-lability of CF, it was felt that oral administration might cause this compound to be less effective against aminopterin toxicity. However, results indicated that levels of CF as low as 1 mg per kilogram of diet were at least partially effective in preventing the toxic symptoms (table 2, groups 1–3). At a level of 5 mg of CF per kilogram of diet, essentially normal growth was obtained and no deaths occurred. In contrast to this, supplements of 100 mg of folacin per kilogram of diet were required to obtain full protection against aminopterin (table 2, groups 6–9). Thus, on the basis of oral administration, CF appeared to be 10 times more effective than folacin in preventing the toxic effects of aminopterin in the rat.

Effect of cortisone and ascorbic acid on aminopterin toxicity

Enlarged and extremely darkened adrenal glands were noted in nearly all of the rats dying from aminopterin feeding. Because of the association of ascorbic acid and cortisone with the adrenal glands, studies were made on the influence of these compounds on the toxicity of aminopterin. It should

TABLE 1

Comparative effectiveness of daily injections of folacin or citrovorum factor on the prevention of aminopterin toxicity in weanling rats
(AES RATS)

GROUP NO.	AMINOPTERIN ADDED	INTRAPERITONEAL INJECTIONS	NO. OF RATS	AVERAGE BODY WEIGHT AT [1]			
				0 Wk.	2 Wks.	3 Wks.	4 Wks.
	mg/kg diet	*µg/day*		*gm*	*gm*	*gm*	*gm*
1	0	0	9	49	113 (9)	144 (9)	175 (9)
2	0.75	0	10	44	95 (4)	124 (1)	141 (1)
3	1.0	0	25	48	65 (5)	... (0)	...
4	0.75	40, CF	6	59	133 (6)	167 (6)	193 (6)
5	0.75	40, folacin	6	59	114 (6)	147 (5)	151 (5)
6	1.0	25, CF	6	54	94 (6)	113 (6)	121 (4)
7	1.0	50, CF	8	53	97 (8)	130 (6)	163 (6)
8	1.0	100, CF	12	52	107 (12)	142 (12)	173 (12)
9	1.0	25, folacin	6	53	81 (5)	64 (2)	... (0)
10	1.0	50, folacin	6	53	74 (4)	66 (2)	... (0)
11	1.0	100, folacin	12	54	87 (3)	79 (2)	... (0)
12	1.0	250, folacin	6	57	108 (3)	112 (2)	120 (1)
13	1.0	10, B-12	3	44	... (0)	...	...

[1] Numbers in parentheses represent survivors remaining at time indicated.

be mentioned in this connection that Nichol and Welch ('50a, b) reported evidence that ascorbic acid may be involved in the conversion of folacin to CF. Gaines and Totter ('50) also demonstrated that dehydroisoandrosterone could replace folacin in the nutrition of *S. faecalis* and *Lactobacillus casei*. Cortisone acetate has been shown recently to have a similar effect and, in addition, can replace CF in the nutrition of *Leuc. citrovorum* (Gaines and Broquist, '51; Gaines, Broquist and Williams, '51).

Results from the present study revealed that daily intraperitoneal injections of 250 μg of cortisone acetate were ineffective in preventing the deaths of animals fed aminopterin (1 mg/kg of diet). However, similar injections of cortisone in the presence of suboptimum injections of CF (50 μg/day)

TABLE 2

Comparative effectiveness of dietary supplements of folacin or citrovorum factor on the prevention of aminopterin toxicity in weanling rats

(AES RATS)

GROUP NO.	DIET [1]	NO. OF RATS	AVERAGE WEIGHT AT [2]			
			0 Wk.	1 Wk.	2 Wks.	3 Wks.
			gm	gm	gm	gm
1	Basal	22	51	79 (22)	120 (22)	149 (22)
2	Basal + aminopterin	24	50	63 (24)	67 (3)	... (0)
3	Basal + aminopterin + 1 mg CF	10	58	82 (10)	82 (5)	96 (5)
4	Basal + aminopterin + 5 mg CF	18	55	83 (18)	119 (18)	137 (18)
5	Basal + aminopterin + 10 mg CF	14	53	82 (14)	122 (14)	151 (14)
6	Basal + aminopterin + 10 mg folacin	10	51	74 (10)	93 (5)	97 (3)
7	Basal + aminopterin + 25 mg folacin	10	50	72 (10)	96 (7)	127 (5)
8	Basal + aminopterin + 50 mg folacin	5	50	80 (5)	109 (4)	132 (4)
9	Basal + aminopterin + 100 mg folacin	10	58	94 (10)	137 (10)	162 (10)

[1] When indicated, aminopterin was added at a level of 1 mg/kg of diet. The amounts indicated for citrovorum factor (CF) and for folacin are the amounts added per kilogram of diet.

[2] Numbers in parentheses represent survivors at time indicated.

appeared to afford some protection. Deaths in the animals were prevented during the 5-week experimental period, but the treatment was ineffective in preventing the anemic condition and only partially effective in preventing the growth inhibition associated with aminopterin feeding. When folacin (200 μg/day) and cortisone (250 μg/day) were injected simultaneously, no protection was obtained. Daily injections of 10

mg of ascorbic acid were ineffective, alone or in association with injections of folacin, in preventing the lethal effects of aminopterin. In other experiments it was noted that injections of ascorbic acid also failed to enhance the effectiveness of dietary folacin.

Effect of antibiotics on aminopterin toxicity in the rat

Previous experiments in this laboratory demonstrated that antibiotics may spare or reduce the requirements of rats for certain B vitamins (Sauberlich, '52a). A similar observation has been made by other investigators in the case of the rat (Lih and Baumann, '51; Linkswiler, Baumann and Snell, '51) and the chick (Biely and March, '51). In view of these observations, the possibility existed that antibiotics, through a similar effect on folacin or CF, might protect against the effects of aminopterin. Waisman and co-workers ('51) reported that aureomycin could counteract the effects of aminopterin in the rat. Repeated experiments in this laboratory, using penicillin or aureomycin (100 mg/kg of diet), have failed to demonstrate any protective effect of antibiotics for two strains of rats fed aminopterin (1 mg/kg of diet). The rats developed the usual symptoms, such as poor growth, anemia and leukopenia, and died. Results were essentially the same regardless of whether the AES strain or the SD strain of rats was used. Daily injections of 100 μg of CF afforded protection to both strains of animals.

Blood studies of rats fed aminopterin

Blood studies were made on all rats at several intervals throughout the experimental period. The studies included hemoglobin analyses, erythrocyte and leukocyte counts, differential determinations and, occasionally, platelet counts. Such studies were indicated, since it was observed that frequently animals partially protected from aminopterin developed changes in the blood as the only readily observable symp-

tom of the toxicity. Some representative data obtained during a 21-day experimental period are presented in table 3.

From the results it may be noted that aminopterin produced a severe reduction both in hemoglobin and in the num-

TABLE 3

Blood studies of rats fed aminopterin

(AES weanling rats; 21-day experimental period)

GROUP NO.	DIET AND SUPPLEMENTS	NO. OF RATS	Hb VALUE [1]	AVE. CELLS PER MM³	
				RBC [1]	WBC [1]
			gm/100 ml		
1	Basal	10	12.3	5,223,000	12,490
2	Basal + aminopterin (4A-PGA) [2]	(5)	(7.0)	(1,995,000)	(2,270)
3	Basal + 4A-PGA + 25 μg CF injected [3]	5	6.0	2,882,000	7,360
4	Basal + 4A-PGA + 50 μg CF injected	4	11.4	4,527,000	9,280
5	Basal + 4A-PGA + 100 μg CF injected	20	11.7	4,538,000	10,180
6	Basal + 4A-PGA + 25 μg folacin injected	2	3.3	743,000	1,800
7	Basal + 4A-PGA + 250 μg folacin injected	2	7.7	3,865,000	7,800
8	Basal + 4A-PGA + 5 mg CF/kg diet	9	11.4	4,522,000	8,140
9	Basal + 4A-PGA + 10 mg CF/kg diet	10	13.1	5,792,000	11,450
10	Basal + 4A-PGA + 20 mg folacin/kg diet	6	6.2	2,980,000	5,310
11	Basal + 4A-PGA + 100 mg folacin/kg diet	8	13.5	5,097,000	10,780

[1] Hemoglobin content determined by the oxyhemoglobin method; RBC = red blood cells; WBC = white blood cells.

[2] Aminopterin added to diets at a level of 1 mg/kg diet. Values in parentheses were obtained at 8 days as all rats on this diet were dead by 21 days.

[3] All injections were made daily intraperitoneally.

ber of erythrocytes and leukocytes present. Similarly, aminopterin caused a reduction in the number of platelets present, from a normal of over 600,000 to 50,000 (per cubic millimeter) or less. Differential leukocyte counts revealed that the

feeding of aminopterin produced essentially a complete absence of neutrophils and the presence of only lymphocytes, in contrast to the normal presence of 15 to 30% neutrophils and the remainder as lymphocytes with a few scattered numbers of eosinophils, basophils and monocytes.

The use of increasing amounts of CF, injected (25 to 100μg/day) or fed (5 to 10 mg/kg diet), progressively reversed the toxic effects noted in the blood (table 3). Injected amounts of folacin up to 250 μg per day were only partially effective, but oral feeding of folacin at a level of 100 mg per kilogram of diet afforded essentially full protection.

Effect of aminopterin in enzyme studies

Dinning, Keith and Day ('51) observed a disturbed transmethylation reaction in chickens deficient in folacin. Williams ('51a) also observed that both aerobic and anaerobic methylation of homocysteine by rat liver homogenates were decreased significantly by dietary aminopterin. Similar experiments were performed in this laboratory to determine the effect of dietary CF and folacin in transmethylation studies *(in vitro)* with rats fed aminopterin.

In preliminary studies it was observed that the most satisfactory experimental conditions were obtained with the use of rat liver homogenates in Ringer's phosphate buffer (pH 7.5) together with DL-homocysteine and choline or betaine. The addition of supplements of adenosine triphopshate, cytochrome C, coenzyme I, vitamin B_{12}, folacin or CF to the reaction mixture failed to enhance methionine formation from homocysteine in the presence of either betaine or choline. Additions of a neutralized solution of ascorbic acid appeared to be somewhat inhibitory. Methionine formation was somewhat less when liver slices were used in place of homogenates, or when DL-homocystine was used in place of DL-homocysteine, or when bicarbonate buffer (pH 7.8) was used in place of Ringer's phosphate buffer.

Liver homogenates (2.25 gm tissue + 10 ml buffer) of the experimental animals were prepared in Ringer's phosphate

buffer and 2.5-ml aliquots placed in beakers of a Dubnoff metabolic shaking apparatus. Additions of 0.5 ml of 0.0234 M (3.16 mg/ml) DL-homocysteine hydrochloride solution prepared with Ringer's phosphate buffer were made. In certain beakers 0.1 ml of a neutralized 0.128 M (20 mg/ml) choline chloride or 0.128 M (19.7 mg/ml) betaine hydrochloride solution was added. All beakers were made to a constant volume with distilled water and incubated 4 hours at 37°C. The reactions were stopped with heat and the contents analyzed for methionine microbiologically with the use of *Leuc. citrovorum* and *Leuc. mesenteroides P-60*. Supplementary experiments revealed that these organisms responded specifically to methionine. Some of the findings thus obtained are presented in table 4.

The results indicated that the formation of methionine from homocysteine in the presence of betaine was about twice that obtained in the presence of choline. The toxic condition induced by aminopterin caused a marked reduction in methionine formation in the presence of either choline or betaine (table 4, groups 1 and 2). These results appear to be in agreement with the similar findings of Williams ('51b). The addition of 10 mg of CF to the diet appeared to prevent the reduction in transmethylation reactions caused by aminopterin. Dietary folacin was also effective, but at a much higher level (100 mg/kg diet). The severity of the aminopterin inhibition in the animals is reflected in the low hemoglobin values obtained (table 4).

Supplementary studies

In view of the changes noted in the adrenal glands of animals fed aminopterin and the association of ascorbic acid with this organ, determinations for this vitamin were made in several series of rats. Ascorbic acid was determined in the liver and the adrenal glands by the photometric indophenol-xylene procedure, based on the method of Robinson and Stotz (Gyorgy, '50). The weanling rats were on the experimental diets for a period of two weeks before the analyses were made.

From the results of the experiments it was observed that the inclusion of 1 mg of aminopterin per kilogram of basal diet caused a reduction in liver ascorbic acid concentration from a normal of 0.38 mg/gm wet tissue to 0.28 mg/gm of liver. This reduction was not as severe as that reported by Williams ('51a) in a similar experiment with adult rats. The reduced concentration of liver ascorbic acid caused by the

TABLE 4

Methionine formation from DL-*homocysteine by homogenates of liver from rats fed aminopterin*

(Weanling SD rats; 21 days on experiment)

GROUP NO.	DIET [1]	NO. OF RATS	Hb VALUES [2]	METHIONINE FORMED [3] (mg/gm wet liver)	
				Substrate	
				Betaine	Choline
			gm/100 ml		
1	Basal	3	12.4	0.91	0.41
2	Basal + aminopterin	4	4.8	0.65	0.26
3	Basal + aminopterin + CF (5 mg)	1	8.6	0.78	0.49
4	Basal + aminopterin + CF (10 mg)	4	12.8	0.93	0.45
5	Basal + aminopterin + folacin (50 mg)	2	6.3	0.65	0.30
6	Basal + aminopterin + folacin (100 mg)	4	12.7	0.78	0.44

[1] Aminopterin added at a level of 1 mg/kg diet. Amounts indicated for citrovorum factor (CF) and folacin were added per kilogram of diet.

[2] Hemoglobin values determined by the oxyhemoglobin method.

[3] Values are corrected for blank formation; incubation period 4 hours at 37°C., using a Dubnoff metabolic shaking incubator.

aminopterin feeding was prevented by the addition of 10 mg of CF or 100 mg of folacin per kilogram of diet. The ascorbic acid concentration of the adrenals was reduced by aminopterin feeding from 4.22 mg/gm wet tissue to 2.81 mg/gm. However, the adrenal glands of the rats fed aminopterin were about 30% greater in weight than those of control animals. Thus, the ascorbic acid content per organ was nearly the same for the two groups of animals. With supplements to the aminopterin diet of CF (10 mg/kg diet) or folacin (100

mg/kg diet), the ascorbic acid concentration of the adrenals was 3.57 mg/gm and 3.88 mg/gm, respectively.

Schaefer and co-workers ('50, '51) and Strength, Schaefer and Salmon ('51) reported that the inclusion of folacin in the diet produces a sparing effect on the choline requirement of weanling rats as demonstrated by increased growth and the prevention of renal damage. Similar experiments in this laboratory revealed that CF was fully as active as folacin administered under the same conditions in demonstrating this effect. The CF was effective either fed in the diet (2 mg/kg diet) or administered by daily intraperitoneal injections (20 μg).

In a final experiment, high protein diets (50% casein) failed to prevent the toxic effects of aminopterin and appeared possibly to aggravate the condition.

DISCUSSION

From the results presented, it is apparent that the synthetic citrovorum factor (leucovorin) is approximately 10 times more effective than folacin in preventing the toxic effects of aminopterin in rats. However, Stokstad ('52) reported that leucovorin is a racemic mixture of diastereoisomers, with the one isomer biologically inactive. Thus, the activty of CF in counteracting the effects of aminopterin may be considerably greater or possibly twice that noted in the present study.

Although CF is considered an acid-labile compound, its oral administration appeared to be as effective as an equivalent amount injected. However, it is possible that the injected amounts of CF would have been more effective if administered in several divided injections each day instead of in a single daily dose.

Considerable evidence exists to indicate that folacin is converted to CF in the body (Sauberlich, '49b, '52b; Nichol and Welch, '50a, b; Broquist, Stokstad and Jukes, '51). In view

of this evidence, together with the observed greater activity of CF in reversing aminopterin inhibition, it may be that CF is the metabolically active form of folacin.

Although cortisone and dehydroisoandrosterone can replace folacin in the nutrition of certain bacteria, rather large amounts are required. This failure to counteract aminopterin may be related to low folacin activity. In the presence of some CF, thereby creating a less drastic condition, cortisone had a slight effect. The use of larger amounts of cortisone might have enhanced this protection; however, this is counter-indicated, since, even at the levels used, there was a slight inhibitory effect on the growth of animals.

Various reports indicate that ascorbic acid may be involved in the conversion of folacin to CF (Nichol and Welch, '50a, b; Williams, '51a; Broquist, Stokstad and Jukes, '51). However, in the present study, ascorbic acid did not prevent the toxic effects of aminopterin. Similarly, the sparing action of antibiotics on vitamins was also insufficient to prevent the effects of aminopterin. It is possible that with the use of lower levels of aminopterin, a protective effect could be demonstrated with ascorbic acid and antibiotics. The total ascorbic acid content of the adrenal gland was not changed by aminopterin feeding, but, due to a hypertrophy of the glands, there was a decrease in the concentration of this factor. There was also a decreased concentration of liver ascorbic acid.

The observation that aminopterin decreased methionine synthesis *in vitro* may implicate folacin in transmethylation reactions in the rat. Such a suggestion has been made by Williams ('51b). In evaluating the data on enzyme activity and ascorbic acid concentrations, consideration must be given to the possibility that the changes may have been a result, in part, of the hemorrhagic necrosis observed to be present in liver, adrenal glands and other tissues of rats fed aminopterin (Copeland and Sauberlich, '53).

SUMMARY

1. Aminopterin (4-aminopteroylglutamic acid) fed at a level of 0.75 to 1.0 mg/kg diet was highly toxic to weanling rats, resulting in poor growth, anemia, leukopenia and death. Daily injections of 25 μg of synthetic citrovorum factor (leucovorin) afforded partial protection, while full protection was obtained with 100-μg injections. In contrast, daily injections of 250 μg of folacin were essentially ineffective.

2. When fed in the diet, the synthetic citrovorum factor (CF) appeared to be 10 times more effective than folacin in preventing the toxic effects of aminopterin. Full protection was afforded by 5 to 10 mg of CF or by 100 mg of folacin per kilogram of diet.

3. Injections of ascorbic acid alone or with folacin were ineffective against aminopterin. Similarly, injections of cortisone alone or with folacin were ineffective. However, injections of cortisone in the presence of limiting amounts of CF appeared to have some protective effect.

4. Dietary supplements of aureomycin or penicillin appeared to afford no protection against the toxicity.

5. Aminopterin feeding caused a reduction in the ability of liver to perform transmethylation reactions. Dietary CF (10 mg/kg diet) or folacin (100 mg/kg diet) prevented this reduction. The ascorbic acid concentrations of livers and of adrenal glands were determined.

6. CF was fully as effective as folacin in lowering the choline requirement of the weanling rat.

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NUTRITIONAL DEFICIENCIES IN MICE CAUSED BY STRESS AGENTS

DAVID K. BOSSHARDT AND JESSE W. HUFF

Department of Biochemistry, Research Division, Sharp and Dohme, Inc., West Point, Pennsylvania

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The imposition of a nutritional or physiological stress has been shown by numerous investigators to alter animals' requirements for certain known and unknown accessory food factors. The stress agents may function by impeding the growth of intestinal organisms which produce substances essential for the animal's metabolic processes, by increasing the basal metabolic rate of the animal so that the normal supply of accessory food factors is inadequate, or by interfering with an essential nutrient in some specific metabolic process.

It has been shown (Bosshardt, Paul, Barnes and Huff, '50b) that the addition of succinylsulfathiazole (SST) to diets low in fat caused a growth retardation in mice. This growth retardation could be prevented if to the diet were added such materials as fat, cottonseed meal, or rolled oats. Based on these observations, it was tentatively concluded that cottonseed and rolled oats contained a factor, or factors, synthesized by intestinal organisms susceptible to SST, that is required by the mouse for lipid synthesis.

The present study was undertaken to investigate further the original observation and also to study the effects of other stresses.

EXPERIMENTAL

The basal diet employed in all studies consisted of 0.5% corn oil,[1] 20% casein,[2] 20% glucose,[3] 4% salt mixture (Jones and Foster, '42), and 2% cellulose.[4] Each diet was supplemented to contain, per 100 gm, 4 mg of α-tocopherol, 900 U.S.P. units of vitamin A, 180 U.S.P. units of vitamin D, 1 mg of 2-methyl-1,4 naphthoquinone diacetate, 0.8 mg of thiamine hydrochloride, 1.6 mg of riboflavin, 0.8 mg of pyridoxine hydrochloride, 4.0 mg of niacin, 4.4 mg of calcium pantothenate, 4.0 mg of para-aminobenzoic acid, 200 mg of choline chloride, 20 mg of inositol, 0.2 mg of folacin, 0.02 mg of biotin, and 0.03 mg of vitamin B_{12}. The remainder of each diet consisted of white dextrin, the level of which was varied when additional supplements were added.

Preliminary studies employing the SST-containing low fat basal diet showed that when the dietary level of a partially defatted cottonseed meal[5] or of an hydrogenated cottonseed oil[6] was varied, a straight line log dose-response curve was obtained. It was therefore decided to use the partially defatted cottonseed meal and the hydrogenated cottonseed oil as primary standards. The interpretation of results obtained would then be based on the assumption that fat per se is required by the growing animal. Fat, if not supplied in adequate quantities in the diet, must then be synthesized by the animal. In order for the animal to be able to synthesize adequate quantities of fat, a factor, or factors, synthesized by intestinal microorganisms and also present in cottonseed, must be supplied.

Male albino weanling mice[7] were used in these studies. All diets were fed ad libitum to groups of 8 mice that were

[1] Mazola, Corn Products Refining Co., Argo, Ill.
[2] Vitamin-test, General Biochemicals, Inc., Chagrin Falls, Ohio.
[3] Cerelose, Corn Products Refining Co., Argo, Ill.
[4] Cellu flour, Chicago Dietetic Supply Co., Chicago, Ill.
[5] Proflo, Traders Oil Mill Co., Fort Worth, Texas.
[6] Primex, Proctor and Gamble, Cincinnati, Ohio.
[7] Obtained from the Beverly Farms, Riverside, New Jersey.

housed in individual wire bottom cages. The test periods were 7 or 14 days, depending on the nature of the stress employed.

The stresses employed, the rationale for their use, and the results obtained will be discussed.

Studies with antibacterial agents

Antibacterial agents may inhibit the growth of certain strains of intestinal microorganisms capable of producing, as a product of their metabolism, a factor, or factors, required by the growing mouse for an essential function, in this case the synthesis of lipid materials. The drugs studied were SST, atabrine,[8] and a combination of SST and dihydrostreptomycin undecylenate.[9]

The results obtained with SST have been previously reported (Bosshardt, Paul, Barnes and Huff, '50b).

An intestinal bacteriostatic system more efficient than SST, namely a mixture of SST and dihydrostreptomycin undecylenate, was investigated. The addition of SST and streptomycin to the low fat basal diet resulted in a more pronounced growth retardation than could be obtained with SST alone. The results, shown in table 1, suggest that the mixture of SST and streptomycin affects the same system as does SST alone, since the growth retardation can be prevented by the addition to the basal diet of cottonseed meal or fat.

Atabrine, another bacteriostatic agent, was also studied as an orally administered stress. Again, as shown in table 1, a growth retardation resulted when atabrine was fed. This growth retardation was partially prevented when cottonseed or fat was included in the diet. It would appear from the data in table 1 that atabrine produces a growth suppression that is not completely overcome by cottonseed or fat, as contrasted with the effect of SST or SST plus streptomycin.

[8] Atabrine, Quinacrine-HCl, Winthrop-Stearns, Inc., New York, N. Y.

[9] Dihydrostreptomycin Undecylenate, Merck, 500 gm equivalent to 255 gm dihydrostreptomycin base.

TABLE 1

The effects of cottonseed and fat on the growth retardations in mice caused by antibacterial agents

DIETARY SUPPLEMENT	AVE. 7-DAY WT. GAIN
	gm
None	6.5
1 % SST	4.4
0.2 % Streptomycin [1]	5.4
1 % SST + 0.2% streptomycin	0.1
1 % SST + 0.2% streptomycin + 3% cottonseed	4.1
1 % SST + 0.2% streptomycin + 5% cottonseed	5.3
1 % SST + 0.2% streptomycin + 10% cottonseed	6.2
1 % SST + 0.2% streptomycin + 5% fat	1.9
1 % SST + 0.2% streptomycin + 15% fat	5.1
1 % SST + 0.2% streptomycin + 25% fat	6.4
0.05% Atabrine	2.5
0.05% Atabrine + 5% cottonseed	3.8
0.05% Atabrine + 15% cottonseed	3.5
0.05% Atabrine + 25% fat	4.4
2 % SST	1.8
2 % SST + 5% cottonseed	5.2
2 % SST + 10% cottonseed	5.9
2 % SST + 25% fat	5.8
2 % SST + 2% 1: 20 liver	— 0.3
2 % SST + 2% 1: 20 liver + 3% cottonseed	1.7
2 % SST + 2% 1: 20 liver + 6% cottonseed	3.5
2 % SST + 2% 1: 20 liver + 10% cottonseed	6.7
2 % SST + 2% 1: 20 liver + 15% fat	5.6
2 % SST + 2% 1: 20 liver + 25% fat	6.2
2 % SST + 2% 1: 20 liver + 1% extracted liver residue	1.4
2 % SST + 2% 1: 20 liver + 5% extracted liver residue	3.3
2 % SST + 2% 1: 20 liver + 10% extracted liver residue	4.5
5 % 1: 20 Liver	5.3
5 % 1: 20 Liver + 10% cottonseed	5.9
10 % 1: 20 Liver	1.8
10 % 1: 20 Liver + 10% cottonseed	4.0
2 % SST + 2% 1: 20 liver + 15% oleic acid	5.2
2 % SST + 2% 1: 20 liver + 25% oleic acid	2.9
2 % SST + 2% 1: 20 liver + 15% lauric acid	0.4
2 % SST + 2% 1: 20 liver + 25% lauric acid	— 1.4
2 % SST + 2% 1: 20 liver + 15% myristic acid	3.2
2 % SST + 2% 1: 20 liver + 25% myristic acid	4.3
2 % SST + 2% 1: 20 liver + 15% palmitic acid	4.9
2 % SST + 2% 1: 20 liver + 25% palmitic acid	6.3
2 % SST + 2% 1: 20 liver + 15% stearic acid	5.2
2 % SST + 2% 1: 20 liver + 25% stearic acid	6.6

[1] Four-tenths per cent dihydrostreptomycin undecylenate.

Studies with metabolic accelerators

The basal metabolic rate of an animal is controlled to a large extent by the hormone of the thyroid gland. The administration to an animal of thyroglobulin, the thyroid hormone, or thyroxine, the amino acid peculiar to the thyroid hormone, will cause an increase in the basal metabolic rate. An increase in the basal metabolic rate causes an increase in the animal's requirements for certain essential nutrients, notably the energy sources, and also some of the accessory food factors necessary for the production of energy from foodstuffs. In this regard it has been well established that the administration of thyroid-active materials to animals increases their requirements for certain of the B vitamins.

If the present problem involves a factor (or factors) that is involved in a metabolic process, it should be possible by the administration of a thyroid-active material to increase the animal's requirement for the postulated factor to a level whereby the animal's normal sources of supply, from food and as a result of synthesis by intestinal microorganisms, are not adequate, so that deficiency symptoms become evident.

A commonly used source of thyroxine is an artificially iodinated casein with thyroid activity.[10] This material has been employed (Bosshardt et al., '49, '50a) to create a deficiency in mice of vitamin B_{12} and was used as the basis for a mouse growth assay for vitamin B_{12}.

Iodinated casein was used in the present study as a stress agent added to the basal low fat diet. The feeding of iodinated casein resulted in a growth retardation. The results, shown in table 2, show that this growth retardation could be prevented by the feeding of cottonseed or fat.

To eliminate the possibility that the iodinated casein was affecting the intestinal microorganisms, a similar experiment was performed except that the thyroid-active material, instead of being fed, was given by intraperitoneal injection. In this study, crystalline DL-thyroxine was used.

[10] Protamone, Cerophyl Laboratories, Kansas City, Mo.

The data in table 2 show a slight growth retardation due to the injected thyroxine. This growth retardation not only was prevented by the feeding of cottonseed and fat, but also it was found that the feeding of cottonseed in conjunction with injected thyroxine resulted in a growth rate considerably above that of the controls. This is in conformity with the results of Koger and Turner ('43), who observed a growth stimulation when mice were given small doses of thyroid.

TABLE 2

The effect of cottonseed and fat on thyroxine-induced growth retardations in mice

DIETARY SUPPLEMENT	AVE. 14-DAY WT. GAIN
	gm.
None	10.6
1% Protamone	4.7
1% Protamone + 5% cottonseed	7.1
1% Protamone + 10% cottonseed	10.9
1% Protamone + 20% cottonseed	10.8
1% Protamone + 25% fat	8.5
3% Protamone	2.1
3% Protamone + 20% cottonseed	9.3
Thyroxine [1]	8.5
Thyroxine + 5% cottonseed	12.9
Thyroxine + 15% cottonseed	13.2
Thyroxine + 25% fat	10.0

[1] Thyroxine = 0.16 mg DL-thyroxine administered by intraperitoneal injection three times per week.

Of necessity, this growth-stimulating effect will manifest itself only when the remainder of the diet is complete with regard to all required nutrients.

Studies with metabolic inhibitors

It has been suggested that extracts of liver contain an antagonist or anti-metabolite for the postulated factor, or factors, in cottonseed (Bosshardt et al., '50b). It has since been found that whole liver shows no effect when fed with a SST-containing low fat diet or may, in fact, be inhibitory, and that

water extracts of liver fed under similar conditions are very toxic. However, the water-extracted liver residue was found to prevent completely the growth retardation caused by SST.

In table 1 are shown the results obtained with liver and SST. When fed in conjunction with SST, liver powder,[11] a water-soluble fraction of liver, caused a greater growth retardation than did SST alone. Although both cottonseed and fat were effective in partially preventing this growth retardation, equivalent levels were not as effective against the combined stress as against SST alone. Water-extracted liver residue was also found to be partially effective against the combined stress of SST and 1:20 liver powder. Although relatively high levels are required, 1:20 liver powder by itself will also cause a growth retardation that can be prevented by the simultaneous feeding of cottonseed.

It may be postulated from these results that whole liver contains in a water-insoluble form the factor, or factors, required to overcome the stresses studied, and contains also in a water-soluble form a metabolic inhibitor, or inhibitors, for the active material.

Further studies on the role of fat in overcoming the effects of the various stress agents were carried out, employing a basal diet with the combined SST and 1:20 liver powder stress.

If the role of fat is to supply preformed long chain fatty acids for the animal, thus decreasing the animal's requirements for synthesis, it should be possible for pure fatty acids to prevent the growth retardation due to the stresses studied. This problem was investigated and some of the results obtained are shown in table 1. The activity shown by the long chain fatty acids, oleic, myristic, palmitic, and stearic, lend credence to the idea that the role of fat is primarily to supply fatty acids required by the animal. If the fatty acids are not supplied preformed, it is necessary for the animal to synthesize them. It then would follow that the non-fat materials that are effective in preventing the apparent fat deficiency

[11] Wilson's 1:20.

symptoms supply a factor, or factors, required by the animal for the synthesis of fat from other foodstuffs.

Studies involving a metabolic imbalance

The administration of a metabolic stress such as thyroid can increase the over-all metabolic rate so that the normal supply of certain accessory nutrients is inadequate to supply the animal's needs. It should likewise be possible to over-

TABLE 3

The effect of cottonseed and a water-extracted liver residue on the growth of normal mice and on mice fed SST and triacetin

DIETARY SUPPLEMENT	AVE. 12-DAY WT. GAIN
	gm
None	7.3
2% SST	5.7
2% SST + 3% cottonseed	7.4
15% Triacetin	2.6
15% Triacetin + 10% cottonseed	7.2
15% Triacetin + 2% SST	2.9
15% Triacetin + 2% SST + 1% cottonseed	5.6
15% Triacetin + 2% SST + 3% cottonseed	5.0
15% Triacetin + 2% SST + 5% cottonseed	7.1
15% Triacetin + 2% SST + 10% cottonseed	6.8
15% Triacetin + 2% SST + 15% cottonseed	8.2
25% Fat	9.1
25% Fat + 5% cottonseed	10.3
25% Fat + 10% cottonseed	11.1
25% Fat + 15% cottonseed	11.5
25% Fat + 20% cottonseed	13.1
25% Fat + 5% water-extracted liver residue	11.6

load the system with a metabolic precursor to such an extent that it cannot be completely metabolized by the organism and thus may become a toxic agent.

In the study to be reported, triacetin was used as a fat precursor. Acetate can be utilized by the organism in several ways, one of which is as a precursor of fat. Unutilized acetate can, if present in large enough concentration, produce toxic effects in the animal by upsetting the acid-base balance. In table 3 are shown some of the results obtained with the feed-

ing of various combinations of triacetin, SST and cottonseed. The growth retardation produced by SST and triacetin was no greater than that produced by triacetin alone, both treatments being more severe than SST alone. These growth retardations were preventable by the feeding of cottonseed.

Studies involving no stress

The data obtained in the studies using injected thyroxine suggested that the basal diet employed in this experiment was inadequate for optimum growth of mice.

For this portion of the study a basal diet containing 25% fat [12] was employed. Using this diet, it was found that both cottonseed and a water-extracted liver residue exerted a beneficial effect on the growth of mice. The results obtained are shown in table 3. It would appear from these data that the basal diet employed in these studies is lacking in an essential nutrient, or nutrients, that is present in cottonseed and liver.

DISCUSSION

Under proper dietary conditions it is possible to produce apparent nutritional deficiencies in growing animals by the administration of various stress agents. These stress agents function by altering either the animal's supply of, or requirement for, certain essential nutrients. The administration of appropriate stress agents can increase the animal's dietary requirement for known accessory growth factors, notably the B vitamins, or, of more recent interest, for as yet unidentified nutrients. It has been shown, for example, by Ershoff ('47, '48) that a water-extracted liver residue is beneficial in preventing the deleterious influence on the growth of young rats resulting from the feeding of thyroid or atabrine. Ershoff ('49) has also shown that fat exerts a beneficial effect on the growing hyperthyroid rat.

Bosshardt, Paul, Barnes and Huff ('50b) have shown that fat and a partially defatted cottonseed meal are effective in

[12] See footnote 6, page 118.

preventing the growth retardation of mice caused by the addition of SST to a low fat diet. It was tentatively concluded that fat per se is an essential nutrient which, if not supplied in the diet, must be synthesized by the animal, and that cottonseed contains a factor, or factors, essential for this synthesis. These results have been confirmed in the present study.

Various stress agents with different postulated mechanisms of action were investigated in this study. In all cases it was found that their deleterious effects on the growth of mice could be prevented by the feeding of fat or a partially defatted cottonseed meal. Although it was not tested against all of the stresses studied, a water-extracted liver residue was active where it was investigated. The results obtained with the long chain fatty acids indicate that the activity of fat is in providing a metabolic end product, rather than as a carrier for an as yet unidentified accessory food factor. The activity obtained with the partially defatted cottonseed meal and the water-extracted liver residue cannot be explained on the basis of their fat contents.

Huff and Bosshardt ('52) have reported that purines were partially effective in preventing the growth retardation resulting from the addition of SST to a low fat diet. This work is continuing with the other stresses described in this report.

SUMMARY

Feeding to growing mice of a low fat diet complete with regard to all known dietary essentials results in what is regarded as normal growth. The addition to this diet, however, of such metabolic stresses as are brought about by the intestinal bacteriostatic agents, succinylsulfathiazole, streptomycin or atabrine, or such materials as thyroid or triacetin, causes a retardation of growth. This growth retardation can be prevented if to the stress-containing basal diet are added fat, fatty acids, or such low fat materials as a partially defatted cottonseed meal or a water-extracted liver residue. It has been concluded tentatively that the stresses employed

interfere with the synthesis of long chain fatty acids by the organism. It is further suggested that cottonseed and liver contain a factor, or factors, essential for the synthesis of fat by the animal.

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THE EFFECT OF TERRAMYCIN ON THE GROWTH AND BODY COMPOSITION OF PIGS[1,2,3]

TEH-CHENG HUANG AND CLIVE M. McCAY
Department of Animal Husbandry, Cornell University, Ithaca, New York

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INTRODUCTION

Since Stokstad et al. ('49) and Jukes et al. ('50) first explored the growth-promoting effect of antibiotics on chicks and pigs, much additional work has been done on this effect. Nevertheless, the mode of action of antibiotics in promoting growth and improving feed utilization in animals remains unknown. Linkswiler et al. ('51), Lih and Baumann ('51), and Biely and March ('51) noticed that antibiotics were effective only when the animal rations were deficient in certain vitamins. Sieburth et al. ('51) observed that terramycin reduced the toxin-producing bacteria, *Clostridium perfringens,* in pig intestines. Recent work by Wahlstrom et al. ('52) failed to disclose such bacteria in the feces of baby pigs fed no antibiotics. Others postulated that antibiotics reduce certain intestinal bacteria which compete with the host for nutrients, or promote the growth of beneficial bacteria which produce certain unknown nutrients for the host.

Bowland et al. ('51) found that antibiotics impaired the quality of meat by increasing fat deposition in the carcass.

[1] This research was supported by the China Institute in America, New York, New York.

[2] The terramycin used in this experiment was a gift from Charles Pfizer and Company, New York. The vitamin B_{12} was given by Merck and Company, Rahway, New Jersey.

[3] The authors are indebted to Professor B. H. Schneider, Department of Animal Husbandry, State College of Washington, for valuable suggestions regarding the conduct of the experiment.

Increased fat deposition was also observed by Arnrich et al. ('52), who added aureomycin to a purified diet for dogs. However, Catron et al. ('52) were unable to note such an increase of fat in swine.

Welsh et al. ('48) noticed the presence of penicillin in the blood and milk of cows 30 hours after injection of the antibiotic. Hobby et al. ('50) observed that terramycin was detectable in the blood of dogs 24 hours after injection. Such a residue might have some carry-over effect in the meat of animals which receive it in their ration.

The present experiment was designed to determine: (1) the mode of action of terramycin in the growth of pigs, (2) its effect on body composition, and (3) its residual presence in the meat.

PROCEDURE

Twelve litter-mate weanling pigs from sows fed a natural ration were divided according to their size and sex into two lots. Lot I received the basal ration and lot II the basal ration plus terramycin hydrochloride at a level of 33 mg per kilogram of feed. The basal ration had the following percentage composition: ground yellow corn 57.0, soybean oil meal 30.8, alfalfa meal 10.0, limestone 1.0, dicalcium phosphate 0.7, iodized salt [4] 0.5, and vitamin supplement.[5]

The two groups of animals were self-fed in groups on concrete floors. Growth, feed intake, and general physical condition were observed. Digestion trials were run during the 5th and 14th weeks in this experiment, using 1% chromic oxide as the indicator according to the method of Barnicoat ('45).

The pigs were slaughtered when they reached 80 kg body weight. Liver, heart, tongue, and leaf fat were saved, and their specific gravity determined according to the method of Brown et al. ('51). Back fat thickness at the first and last ribs, and the length of the body from the atlas joint to the base of

[4] Containing trace minerals of the following composition (grams per 100 kg of salt): copper sulphate 70, ferrous sulphate 876, cobaltous nitrate 97, and manganese sulphate 7,146.

[5] Vitamin supplement (milligrams per kilogram of feed): calcium pantothenate 9.9, riboflavin 1.8, niacin 11.0, vitamin B_{12} 0.01, and vitamin D 198 U.S.P. units.

the tail, were measured. The right half of the carcass of each pig was separated as follows: lean meat (muscular tissue without fat), mixed meat (in which muscular tissue and adipose tissue were not separable), fat, skin, bone (cooked to facilitate the removal of flesh), and meat scraps (meat from the bone). These materials were finely ground, frozen, and stored in airtight containers.

The percentages of dry matter, protein, fat, and ash in the internal organs and other tissue samples were determined for each individual pig. The internal organs and lean meat were assayed for vitamin A and thiamine. All the determinations were made according to Association of Official Agricultural Chemists methods ('45).

Protein quality of the lean meat from each group was assayed by the rat growth method. Male weanling albino rats were selected as the experimental animals for this phase. Four litter mates were used in each comparison group. The rations for the rats consisted of sugar, lean pork meat, and vitamin supplement.[6] Lard and minerals [7] were added to make all the rations have the same percentage of fat and ash. All the rations had 13% fat, 5% ash, and 7 to 25% protein supplied by the lean pork meat. The rations are shown in table 6.

RESULTS AND DISCUSSION

Growth

Throughout the experiment the pigs receiving the terramycin supplement had significantly better growth than the pigs receiving the basal ration (table 1). The feed consumption of these two groups was the same during the first three weeks. In the meantime, however, the pigs receiving terramycin had

[6] Vitamin supplement (milligrams per kilogram of ration): thiamine 4.4, riboflavin 7.0, niacin 55, calcium pantothenate 44, menadione 22, inositol 220, biotin 0.2, folic acid 4.4, choline chloride 2,200, pyridoxine 13.2, para-aminobenzoic acid 330, and vitamin B_{12} 0.03. Fat-soluble vitamins were fed per rat per day: vitamin A 100 I.U., vitamin D 10 I.U., and vitamin E 0.3 mg.

[7] Mineral mixture, U.S.P. No. XII. All the rations received at least 4.3% of ash from this mixture.

attained an average body weight of 30 kg, compared to 25 kg for the pigs fed the basal ration. This shows that terramycin stimulated the growth of the pigs by improving the feed utilization. During the following weeks the terramycin-fed pigs consumed more feed than the controls, evidently because of their larger size.

Biely and March ('51) and Lih and Baumann ('51) observed that antibiotics are effective in stimulating the growth of chickens or rats only when the basal rations are deficient in certain nutrients. The ration in the present trials was believed

TABLE 1

Results of the pig growth experiment [1]

RATIONS	LENGTH	INITIAL WEIGHT	FINAL WEIGHT	AVERAGE DAILY GAIN	KG OF FEED PER KG GAIN
	days	*kg*	*kg*	*kg*	
Basal	56	14.6	43.3	0.49 ± 0.16	3.09
Basal + terramycin	56	14.6	50.0	0.63 ± 0.16 P<0.01 [2]	2.86
Basal	115	14.6	79.6	0.56 ± 0.08	3.43
Basal + terramycin	96	14.6	79.7	0.68 ± 0.10 P<0.01	3.21

[1] All the results in this table and the following tables represent average of 6 pigs. The figures after the ± signs are standard errors.

[2] "P" represents the probability of chance occurrence of the difference observed between test and control groups, calculated by Student's "t" test.

adequate in all nutritional aspects. Furthermore, it had been fortified by the addition of several B vitamins which Briggs and Beeson ('50) recommended as a supplement in certain pig rations. Nevertheless, the addition of terramycin to this ration significantly promoted the growth of the young pigs. Hence, if the hypothesis of Biely and March is applicable to pigs, these animals must require other nutrients not hitherto recognized as constituents of an adequate ration, or the old recommendation of pig requirements is not adequate. However, other hypotheses with respect to the action of antibiotics in promoting growth should not be overlooked.

Digestion trials

During the 5th week all the digestibility coefficients (table 2) of the different nutrients were higher in the pigs fed terramycin than in the control pigs. The differences in the digestibility of protein and dry matter were the greatest. An analogous improvement of protein utilization was observed by Anderson et al. ('52) in the chick, but not by Black and Bratzler ('52) in the rat. Apparently different species of animals respond differently to antibiotics. During the 14th week the findings were similar to those of the first trial. However, these

TABLE 2

Comparison of digestibility between the two groups of pigs fed rations with or without terramycin using chromic oxide as an indicator

RATIONS	PERIOD	PER CENT DIGESTIBILITY OF THE DIFFERENT NUTRIENTS				
		Dry matter	Protein	Carbohydrate	Fat	Ash
Basal [1]	5th wk.	92.2±1.7	86.9± 4.9 [2]	86.8±5.7	49.6± 8.4	55.1± 7.7
Basal + terramycin	5th wk.	94.4±2.4 P<0.01	92.3± 5.9 P<0.05 [3]	88.6±3.1	53.6±13.1	58.2±11.0
Basal	14th wk.	89.2±8.5	74.8±11.1	88.9±4.9	33.1±24	46.1±18
Basal + terramycin	14th wk.	90.8±4.4	79.8±11.8	86.8±4.1	40.8±39	50.6±20

[1] All the basal rations consisted of the following percentage composition (dry basis): dry matter 88.2, protein 25.2, carbohydrates 65.3, fat 3.0 and ash 6.5.

[2] The figures after the ± signs are standard errors.

[3] "P" represents the probability of chance occurrence of the difference observed between the test and control groups.

values varied so greatly that the differences were not statistically significant.

The pH values of feces and urine, and the indican contents of the urine, were determined in individual pigs for three consecutive days during the first digestion trial. The average pH values of feces or urine were the same for each diet group, namely, 7.6 for the urine and 7.4 for the feces. After standing at room temperature for 48 hours, the feces and urine of both groups had similar shifts of pH. The urine became more alkaline, while the feces changed from alkaline to slightly

acidic. Evidently terramycin, even if present in feces or urine from the pigs fed terramycin, did not play any role in preventing the growth of bacteria. In marked contrast, the excretion of indican was different for these two groups. Both the percentage and total excretion of indican were much higher in the pigs receiving terramycin. The average contents of indican in the urine from the pigs fed terramycin and the basal ration were 106 p.p.m. and 65 p.p.m., while the daily excretions were 11.8 mg and 9.3 mg, respectively.

Carcass studies

The data (table 3) indicate that all the internal organs from the pigs receiving terramycin had a higher specific gravity

TABLE 3

Specific gravity and weights of certain samples from the two groups of pigs

	RATIONS	SAMPLES				
		Heart	Liver	Kidneys	Tongue	Leaf-fat
Specific gravity	Basal	1.038	1.031	1.044	1.059	0.922
	Basal + terramycin	1.049 [1]	1.061 [1]	1.051	1.060	0.930
Weight, gm	Basal	192	1,436	131	152	553
	Basal + terramycin	187	1,276	122	161	632

[1] Average of 5 pigs.

than those from the animals receiving no terramycin. On the other hand, the former had smaller internal organs than the controls.

The dressing percentage was observed to be significantly larger for pigs receiving terramycin (76.1%) than for those fed the basal ration (72.3%). The supplemented animals yielded more total lean meat and fat than the controls, but they had a relatively lower percentage of lean meat and a higher percentage of fat. The former had an average of 18 kg of lean meat (36% of the dressed carcass) and 15 kg of fat (30% of the dressed carcass), while the latter had 19 kg (37%)

and 14 kg (29%), respectively. No difference was noted in the thickness of back fat, in body length, or in bone structure.

Swine fed terramycin had a substantial infestation of ascarids, while the controls were only slightly infested.

Tissue analysis

There were no chemical differences in composition between the hearts, tongues, lean meat, mixed meat, fat, skin, and meat scraps of individuals from the two groups (table 4). The kidneys of the animals receiving terramycin had significantly higher values for protein and fat than those of the animals receiving the basal ration, but this difference is due to the fact that the values for one of the control pigs were all abnormally low. Although terramycin caused an average of 17% more fat deposition in the liver, this difference is not statistically significant.

The whole carcass compositions, based on an analysis of the right halves of the 12 pigs, failed to show any significant differences (table 5). It seems that the composition of the pigs receiving the terramycin was less variable than that of the pigs receiving the basal ration.

The vitamin A and thiamine contents of the two groups of hearts, kidneys, and lean meat were similar (table 4). However, the vitamin A contents were significantly higher in the livers from the animals receiving terramycin.

There was no difference in the iodine numbers and refractive indexes of fat from the two groups. The average iodine numbers of fat from the pigs fed terramycin and the basal ration were 49.1 and 49.6, while the refractive indexes were 1.4579 and 1.4588, respectively.

Biological assay of meat

The data in table 6 show that during the 5-week period there were no growth differences between the groups of rats fed 7, 10 and 13% pork protein. This indicates that the quality of the protein from the two sources of pig meat was similar. All the rats increased in growth with increased levels of protein.

TABLE 4

Chemical composition and vitamin A and B_1 contents of tissue samples from the two groups of pigs fed rations with or without terramycin (wet basis)

COMPOSITION	RATIONS	SAMPLES										
		Heart	Liver	Kidneys	Tongue	Leaf fat	Lean meat	Mixed meat	Fat	Meat scrap	Skin	Bone (cooked)
Dry matter, %	Basal	24.0	29.6	20.1	26.2	92.4	27.6	59.6	88.7	52.6	83.0	73.4
	Basal + terramycin	23.6 [1]	30.3 [1]	22.0	26.7	95.1	28.7	52.9	88.2	50.6	80.1	72.3
Protein, %	Basal	17.5	22.5	14.7	20.7	2.0	17.2	12.8	2.7	26.5	13.7	28.4
	Basal + terramycin	17.8	22.9	16.4	19.9	2.1	18.2	12.9	2.8	24.9	14.2	27.6
Fat, %	Basal	4.2	2.7	3.5	5.8	87.0	11.6	39.1	87.6	25.0	70.5	11.1
	Basal + terramycin	4.5	3.1	4.4	5.9	93.7	11.6	40.2	86.0	25.7	65.8	11.4
Ash, %	Basal	1.0	1.4	1.1	1.0	0.1	1.1	0.6	0.2	1.0	0.2	35.1
	Basal + terramycin	1.0	1.4	1.1	1.0	0.1	1.1	0.8	0.2	1.1	0.3	33.3
Vitamin A μg/gm	Basal	6.6	92	1.8			1.5					
	Basal + terramycin	4.8	136	1.8			1.9					
Thiamine μg/gm	Basal	6.4	15.4	3.8			13.6					
	Basal + terramycin	6.5	17.3	3.8			13.8					

[1] Average of 5 pigs.

However, the addition of the crystalline terramycin to the rat rations, adequate in all respects except for protein, stimulated growth significantly. Terramycin must, therefore, improve the utilization of pork protein in the rat.

TABLE 5

Chemical composition of the edible portion of the pig carcass

COMPOSITION	RATIONS	
	Basal	Basal + terramycin
Dry matter, %	57.5 ± 9 [1]	58.0 ± 10
Protein, %	12.1 ± 2.6	12.7 ± 2
Fat, %	45.7 ± 4.1	46.0 ± 2.7
Ash, %	0.65 ± 0.14	0.69 ± 0.04

[1] The figures after the ± signs are standard errors.

TABLE 6

The growth of rats fed varying amounts of protein from pigs receiving terramycin (5-week study)

RATIONS	INITIAL WEIGHT [1]	FINAL WEIGHT	AVERAGE DAILY GAIN
	gm	gm	gm
7% Basal [2]	50	91.2	1.2
7% Terramycin [3]	50	95.5	1.3
10% Basal	50	116.5	1.9
10% Terramycin	50	112.0	1.8
13% Basal	50	158.5	3.1
13% Terramycin	50	151.8	2.9
13% Basal + terramycin [4]	53	180.9	3.6 $P < 0.01$ [5]
25% Basal	43	157.5	3.3
25% Basal + terramycin	43	192.1	4.0 $P < 0.01$

[1] Average of 4 rats.

[2] "Basal" stands for protein supplied by lean meat from the pigs fed the basal ration.

[3] Protein supplied by lean meat from the pigs fed the basal ration plus terramycin.

[4] Terramycin HCl was added to the ration at a level of 33 mg per kilogram of feed.

[5] "P" represents the probability of chance occurrence of the difference observed between test and control groups.

Welsh et al. ('48) and Hobby et al. ('50) were able to detect penicillin or terramycin in the blood stream of animals 24 hours after an injection. But, since it was noted in the present trial that rats receiving 13% protein had the same growth rate regardless of the source of the lean meat, such trace amounts of terramycin as may be present are not sufficiently large to produce any growth effect in rats.

SUMMARY

1. Terramycin stimulated the growth of pigs fed a corn-soybean basal ration by the improvement of feed utilization from weaning to market weight. This antibiotic improved the digestibility, especially of dry matter and protein, at an early age.

2. When slaughtered at the same market weight (approximately 80 kg), the pigs receiving terramycin had a higher dressing percentage than those receiving no supplement. In gross analysis, the pigs fed terramycin yielded more total fat and lean meat by weight, but a lower percentage of lean meat and a higher percentage of fat than the controls.

3. With the exception of vitamin A content, which was higher in the livers of terramycin-fed pigs, there was no other significant difference in composition between the internal organs or other meat samples from the two groups of animals.

4. Terramycin residues in meat, if any, did not produce any growth effect in rats.

5. Terramycin stimulated the growth of rats fed pork.

6. Terramycin feeding seemed to favor the infestation of swine by ascarids.

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THE EFFECT OF DIETARY AMINO ACID LEVELS UPON THE OXIDATION OF L- AND D-AMINO ACIDS BY KIDNEY TISSUE [1]

A. LEONARD SHEFFNER [2] AND OLAF BERGEIM
Department of Biological Chemistry, University of Illinois College of Medicine, Chicago

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It was previously observed that the free amino acid levels of rat kidney tissue fell to below the normal fasting values several hours after the ingestion of protein (Sheffner and Bergeim, '52). This phenomenon was interpreted as indicating a stimulation of amino acid catabolism resulting from the postprandial higher levels of amino acids in the tissues. Consequently, it seemed of interest to study the effects of diets containing increased quantities of amino acids upon the activity of certain enzymes presumably involved in the oxidation of amino acids.

The results indicated that the rate of oxidation of L-amino acids was increased in kidney tissue by the addition of DL-amino acids to the diet; the rate of oxidation of D-amino acids was increased by amino acid supplementation only when the diet contained suboptimum quantities of protein.

METHODS

Measurement of enzymatic activity in rat kidney

The animal was decapitated and exsanguinated; the kidneys were immediately removed and chilled on cracked ice.

[1] This work was supported by grants from the Nutrition Foundation, Inc., and the University Research Board of the University of Illinois.

[2] Fellow of the National Institute of Health, United States Public Health Service. Present address: Biological Research Laboratory, Southern Illinois University, Carbondale, Illinois.

The tissues were then blotted, weighed on a small torsion balance and homogenized with distilled water in a Potter-Elvehjem glass homogenizer. The procedure of Schneider and Potter ('43) was used for the measurement of succinic dehydrogenase activity. For measurement of the rate of oxidation of L- and D-amino acids the method of Cagan et al. ('50) was used, with modifications to increase the specificity of the procedure. The rate of oxidation of L-amino acids by the kidney supernatants gradually decreased with time. The decrease in activity at 37°C. was essentially the same in the absence of substrate and, therefore, could not be attributed to the formation of toxic products of the amino acid oxidation. The decline in activity followed a first order type of reaction, so that the plot of the log of activity against time was a straight line. Therefore, the oxidation of L-amino acids was recorded as microliters O_2 uptake per 10 mg tissue nitrogen per hour extrapolated to zero time. Optimum activity was found to be at pH 8.5 for both L- and D-amino acids. The substrates were added in excess, but no evidence of toxicity due to substrate was noted. As was done by Cagan et al. ('50), the tissue supernatants were used because of the lower blanks and greater differentiation obtained upon addition of substrate. For comparison of the activity of the kidney tissues during the various dietary periods, L- or D-methionine was used as the substrate. Methionine was used as substrate because it was stable in solution, had very little transaminase activity (Cammarata and Cohen, '50), and gave higher and more consistent values than did the other amino acids tested. In addition, the keto acid of methionine is not rapidly oxidized; with L-methionine as the substrate, the ratio of oxygen atom uptake to moles of ammonia released in kidney supernatants, in the presence of excess catalase, averaged 1.17:1, which is close to the 1:1 predicted by theory for oxidative deamination. Nevertheless, the kidney preparation used here oxidized various L-amino acids at relative rates which were different from that reported by Blanchard et al. ('44) with L-amino acid oxidase;

therefore, it is probable that the oxidation of L-methionine recorded here was due to more than one enzyme.

All experiments were performed with the Warburg apparatus at 37°C., using vessels with two side arms. The Warburg vessel was filled as follows: main compartment — 1.0 ml tissue supernatant (equivalent to 0.20 gm wet kidney tissue) and 1.0 ml 1.25 M sodium pyrophosphate (pH 8.5); side arm 1 — 0.5 ml 0.1 M L- or D-methionine, depending on whether the oxidation of L- or D-amino acids was being measured; side arm 2 — 0.1 ml 6 N H_2SO_4; center well — 0.2 ml of 10% KOH plus filter paper. The methionine solutions were dissolved in 1 N HCl, adjusted with NaOH to pH 8.5, and diluted to a concentration of 0.1 M methionine and 0.15 M NaCl. For the blank determination 0.15 M NaCl was used. Readings were taken every 30 minutes for 90 minutes.

Total nitrogen was determined by the micro Kjeldahl method with cupric sulfate and selenium dioxide as digestion catalysts. Ammonia was measured by aeration of the sample and Nesslerization of the collected ammonia. The concentrations of free amino acids in kidney tissue were determined according to the procedure of Solomon et al. ('51).

EXPERIMENTAL AND RESULTS

Effect of supplemental methionine and phenylalanine upon kidney L- and D-amino acid oxidase activity

Adult white Sprague-Dawley rats were divided into two groups. The first group (4 rats) received a basal diet, while the second (5 rats) received in addition a supplement of DL-methionine which increased the methionine content of the diet from 0.3% to 3%. By supplementing the diet in this manner, the relative concentration of a single amino acid in kidney tissue can be considerably increased postprandially (Sheffner and Bergeim, '52).

The basal diet was of the following percentage composition: vitamin free casein 10, dextrose 25, cornstarch 50.2, peanut oil 11, salt mixture [3] 3, L-cystine 0.3; vitamins included

[3] Hubbell, Mendel and Wakeman salt mixture ('37).

per kilogram were vitamin A 10,000 units, vitamin D 1,000 units, alpha-tocopherol 200 mg, menadione 0.3 mg, riboflavin 8 mg, pyridoxine 8 mg, thiamine 8 mg, calcium pantothenate 16 mg, niacin 25 mg, biotin 0.3 mg, *p*-aminobenzoic acid 300 mg, inositol 2,000 mg, choline hydrochloride 2,000 mg, and folic acid 3 mg.

In order to avoid unequal protein and caloric intake, the animals were individually fed at a level which coincided with the maximum intake of the animals receiving the methionine-supplemented diet. The animals gained a maximum of 35 gm during the 14-day experimental period, due to the limited intake of food.

A second series of experiments similar to the above were performed, the only difference being that 15% casein was included in the basal diet. The animals were divided into three groups: the first group (6 rats) received the basal diet; the second group (4 rats) received, in addition to the basal diet, a supplement of DL-methionine which increased the methionine content of the diet from 0.45% to 3.0%; the third group (5 rats) received the basal diet plus DL-phenylalanine, which increased the phenylalanine content of the diet from 0.77% to 3.85%.

The effect of supplementing the diet with methionine or phenylalanine upon the oxidation of L-methionine in kidney tissue is presented in table 1. The results indicate that the oxidation was significantly increased by supplementation of the diet with either methionine (P[4] $= <.01$) or phenylalanine ($P = <.01$). The postabsorptive free amino acid concentrations in kidney tissues of rats treated identically with those described above indicated (table 1) that a decrease in amino acid levels resulted from methionine supplementation of the diet but not from phenylalanine supplementation.

[4] Tests of significance were calculated from the formulae (Dixon and Massey, '51):

$$t = \frac{\bar{X}_1 - \bar{X}_2}{S_p \sqrt{\frac{1}{N_1} + \frac{1}{N_2}}}; \quad S_p^2 = \frac{N_1 S_1^2 + N_2 S_2^2}{N_1 + N_2 - 2}.$$

The measurement of D-amino acid oxidase activity in kidney tissues indicated (table 2) that supplementation of the 10% casein diet with DL-methionine or increase of the casein content of the diet to 15% resulted in significant increases in D-amino acid oxidase activity. Supplementation of the 15%

TABLE 1

The effect of supplementary DL-*methionine or* DL-*phenylalanine in the diet upon the rate of oxidation of* L-*amino acids and the fasting free amino acid levels in kidney tissue*

(Rate of amino acid oxidation expressed as microliters oxygen uptake per hour per 10 mg tissue nitrogen. The concentrations of amino acids are expressed as micrograms per gram of fresh tissue. The number of animals in each group is indicated in parentheses.)

DIET	L-AMINO ACID OXIDATION		L-METHIONINE CONCENTRATION [3]	L-PHENYLALANINE CONCENTRATION [3]
	Exp. 1 [1]	Exp. 2 [2]		
Control	150 ± 10 [3] (4)	158 ± 12 (6)	3.0 ± 0.7 (4)	95 ± 4.9 (4)
Methionine-supplemented	203 ± 8.0 (5)	246 ± 13 (4)	0.8 ± 0.3 (4)	75 ± 5.4 (4)
Phenylalanine-supplemented		256 ± 23 (5)	2.9 ± 0.6 (4)	113 ± 7.7 (4)

[1] Ten per cent casein basal diet.
[2] Fifteen per cent casein basal diet.
[3] Standard error of the mean.

TABLE 2

The effect of supplementary amino acids in the diet upon D-*amino acid oxidase and succinic dehydrogenase activity*

(Activity expressed as microliters oxygen uptake per hour per 10 mg tissue nitrogen. The number of animals in each group is indicated in parentheses.)

DIET	D-AMINO ACID OXIDASE		SUCCINIC DEHYDROGENASE [2]
	Exp. 1 [1]	Exp. 2 [2]	
Control	220 ± 5.0 [3] (5)	395 ± 37 (6)	5,003 ± 132 (6)
Methionine-supplemented	323 ± 22 (5)	412 ± 47 (4)	4,954 ± 100 (4)
Phenylalanine-supplemented		428 ± 27 (5)	4,900 ± 139 (5)

[1] Ten per cent casein basal diet.
[2] Fifteen per cent casein basal diet.
[3] Standard error of the mean.

casein diet with DL-methionine or DL-phenylalanine produced no change in activity.

Succinic dehydrogenase activity (table 2) was also not affected by supplementation of the 15% casein diet with DL-methionine or DL-phenylalanine.

DISCUSSION

Various dietary factors have been found to influence the activity of D-amino acid oxidase, an enzyme whose apparent substrate has never been found in normal mammalian tissues. For example, the liver and kidney D-amino acid oxidase of rats on a low protein diet decreased with the loss of tissue protein (Seifter et al., '48; Kaplanskii et al., '45). Also, the addition of cystine or thiamine or both to a carbohydrate diet in general stimulated the activity of this enzyme in liver (Vescia and Bartolini, '47). In the present investigation, a study was made of the effects of increasing the ingestion of single amino acids upon the activity of kidney D-amino acid oxidase. In these experiments it was found that increased postprandial tissue levels of single D-amino acids did not result in increased enzyme activity when the animal was maintained upon an adequate diet. However, the addition of DL-methionine or more casein to an inadequate protein diet did cause an increase in the rate of kidney D-amino acid oxidation. The effect of added methionine in the latter case could be interpreted as being due to a limiting factor in the diet being supplied to produce better utilization of the remaining amino acids for non-specific protein synthesis. In this connection, the addition of more protein to the 10% casein diet had no effect upon kidney L-amino acid oxidation, indicating that, for the latter, the increased activity observed upon supplementing the diet with single amino acids was probably a more specific one.

The inhibition by malonic acid of arginine and urea formation (Fahrlander et al., '47; Krebs and Eggleston, '48) as well as succinic dehydrogenase activity indicated that the oxidation of succinate is involved in amino acid catabolism, pos-

sibly as an energy factor. In the present study, no change in succinic dehydrogenase activity was observed in kidneys from rats fed diets supplemented with amino acids.

SUMMARY

1. Supplementation of a 10 or 15% casein diet with DL-methionine or DL-phenylalanine was found to increase the rate of L-amino acid oxidation in rat kidney.

2. The addition of DL-methionine to a 10% casein diet or increase of the casein content of the diet to 15% resulted in significant increases in D-amino acid oxidase activity. Supplementation of the 15% casein diet with DL-methionine or DL-phenylalanine produced no change in activity.

3. Kidney succinic dehydrogenase activity was not altered by supplementation of the diet with amino acids.

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